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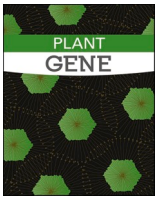

Expression of anthocyanin biosynthesis-related genes during flower development in *Lilium* spp.

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ABSTRACT

Sterility of hybrids produced from interspecific hybridization in lilies (*Lilium*, Liliaceae) is a great limitation in the breeding program, especially for *Lilium longiflorum,* which only has white-flowered cultivars. Because modification of flower colour in L. *longiflorum* by conventional breeding is limited by pre- and post-fertilization barriers, we think genetic modification could be used as an alternative in the future. For this, we need to understand what determines white colouration in L. *longiflorum* and other species and identify the molecular mechanisms regulating flower colour. In this study, we determined the accumulation of anthocyanins and related compounds in flower tissues during flower developmental stages in L. *longiflorum* cultivar 'Lincoln' and in the Oriental hybrid lily cultivars 'Rialto', 'Perth' and 'Gran Tourismo', respectively with white, pink and red flowers. Furthermore, the presence/absence and the expression of eight structural genes (*CHSa, CHSb, CHIa, CHIb, F3H, F3'H, DFR, ANS*) and three transcription factor genes (*MYB12, MYB15, bHLH2*) in flower tissues were investigated. Two structural genes (*LlLinF3'H* and *LlLinDFR*) and one transcription factor gene (*LlLinbHLH2*) were not detected in 'Lincoln' flowers. In 'Rialto', an amino acid substitution in the R2 repeat of *LhRiaMYB12* which was previously reported to be responsible for the white flower colour is also found in the *LhPerMYB12* of the pink 'Perth' flowers. Moreover, *LhRiaDFR* is present but not expressed in 'Rialto' flowers. Accumulation of cyanidin was observed in the flowers of 'Perth' and 'Gran Tourismo'. High amounts of dihydrokaempferol accumulated in flowers of all four lily cultivars confirming the expression and functionality of early structural genes in the pathway. The elevated expression of the structural genes is strongly correlated with the expression of *LhMYB12* and *LhMYB15*. This information can be used in the future to generate new L. *longiflorum* or Oriental lily hybrid cultivars with novel flower colours.

1. Introduction

The genus *Lilium* (family Liliaceae) consists of >100 species that are widely dispersed in Asia, North America and Europe (Comber, 1949; Lighty, 1968; Lim et al., 2008). *Lilium* is classified into sections *Martagon, Pseudolirium, Lilium, Archelirion, Sinomartagon, Leucolirion,* and *Oxypetala* (Comber, 1949; De Jong, 1974), and hybrids within sections *Leucolirion, Archelirion,* and *Sinomartagon* represent the most important groups for breeding (Shahin et al., 2012). They are described as Longiflorum (L), Trumpet (T), Oriental (O) and Asiatic (A) hybrids.

The Oriental and Asiatic hybrids have been commercially predominant in the ornamental cut flower industry due to their outstanding flower shape, fragrance and large colour variation (Lim and Van Tuyl, 2006; Yamagishi and Akagi, 2013). In addition to these two major groups of lilies, *Lilium longiflorum,* together with its cultivars nowadays has become more famous among growers and consumers because of their funnel-shaped flowers and pleasant fragrance, making them hold the third best seller of lily fresh cut flowers in China (Wang et al., 2009). Since this species only has purely white-flowered cultivars, it is believed that varying its flower colour would be of a great potential commercial value. In order to introduce a new flower colour to L. *longiflorum,* a wide range of interspecific crosses between distantly related species, including the crosses in different sections such as LA (Longiflorum x Asiatic) and LO (Longiflorum x Oriental) was done by many researchers

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(Okazaki et al., 1992; Van Tuyl et al., 1997; Chi, 2002; Khan et al., 2009; Wang et al., 2009;). However, F1-hybrids produced by interspecific or intersectional crosses are often sterile (Martens et al., 2003) and it is still difficult to rescue very young embryos and only a few successes have been reported (Wang et al., 2009). Genetic modification offers an alternative for the introduction of new flower colour traits without changing its other positive characteristics and might speed up the breeding program. For this, we need to understand the genetic background of white colouration and the molecular mechanisms regulating flower colour, especially in *Lilium* species.

Pink and red-purple, the predominant flower colours in Oriental hybrid lilies are due to anthocyanin accumulation (Yamagishi and Akagi, 2013), and cyanidin 3-*O*-*β*-rutinoside is the major anthocyanin (Martens et al., 2003). The biosynthetic pathway of anthocyanins has been extensively studied and is conserved among many plant species (Karlov et al., 1999). A simplified anthocyanin pathway from Oriental and Asiatic lilies is presented in Fig. 1. Anthocyanin structural genes (*PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase*) that are involved in anthocyanin biosynthesis have been identified and isolated from lily (Nørbæk and Kondo, 1999; Winkel-Shirley, 2001; Nakatsuka et al., 2003; Liu et al., 2011; Lai et al., 2012). The transcription of anthocyanin biosynthetic genes in lilies is assumed to be regulated by an interaction between *MYB12* and *bHLH2* transcription factors (Suzuki et al., 2015; Nakatsuka et al., 2009; Yamagishi, 2011; Yamagishi et al., 2012). Recently, *LrMYB15* from *Lilium regale* was discovered, which controls anthocyanin pigmentation in the flower buds, leaves and bracts

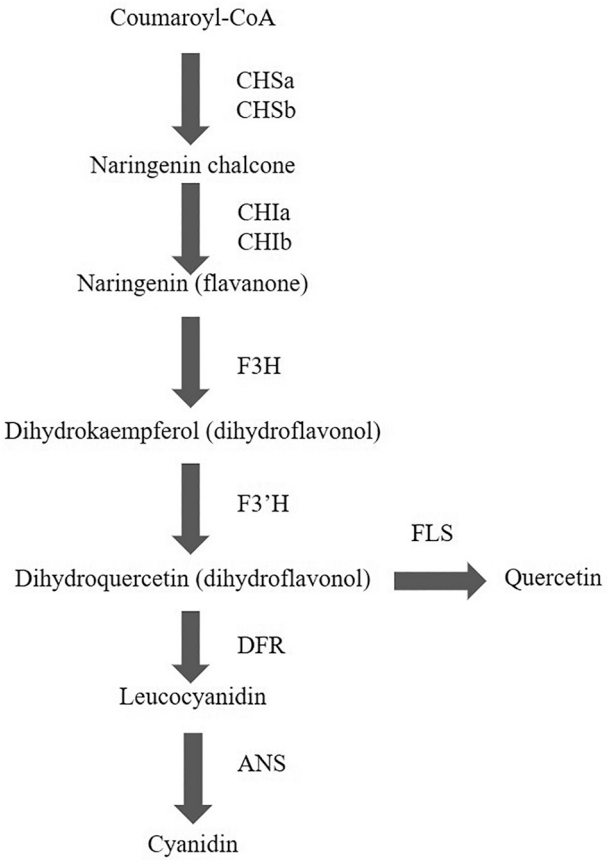

Fig. 1. Simplified anthocyanin biosynthetic pathway in Oriental and Asiatic lilies. *CHSa, chalcone synthase a; CHSb, chalcone synthase b; CHIa, chalcone isomerase a; CHIb, chalcone isomerase b F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3′-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase.*

(Yamagishi, 2016).

Molecular mechanisms regulating white colouration in lilies are variable depending on the plant species being considered. For example, in white-flowered Asiatic hybrid lily cultivars 'Navona' and 'Silver Stone', the expression level of *LhMYB12* was determined and found to be insufficient (Yamagishi, 2011). In the white-flowered Oriental hybrid lily cultivar 'Casa Blanca' and its putative parent *Lilium auratum* var. *platyphyllum,* the *LhMYB12* was present and presumed to be functional, but it was also not expressed in tepals, which might cause the white tepal colour (Yamagishi et al., 2014). In the Oriental hybrid lily cultivar 'Rialto', *LhMYB12* was expressed, but a W-to-L amino acid substitution was detected in the R2-repeat, which likely disrupted its function (Suzuki et al., 2015; Nakatsuka et al., 2009). In the wild species *Lilium speciosum,* the white/red line (i.e. white tepals/red anthers) contained a *LsMYB12* gene that was identical to *LhMYB12* of 'Rialto'. This occurrence was considered to be the cause of the white tepals in the *L. speciosum* white/red line. However, in the white/yellow line (i.e. white tepals/yellow anthers), a nonsense mutation in *LsDFR* was identified which led to a premature stop codon and supposedly caused the white tepal and yellow anther phenotype (Suzuki et al., 2015). These results indicate that there are several mechanisms including mutations, absence of genes, and differential expression levels that generate white flowers in lilies.

In this study, we aimed to investigate the genetic background of two white-flowered lilies, i.e. the L. *longiflorum* cultivar 'Lincoln' and the Oriental hybrid lily cultivar 'Rialto' by examining the expression of anthocyanin biosynthesis-related genes during multiple flower developmental stages. The presence/absence of the genes was demonstrated by gene-specific amplification on complementary DNA and genomic DNA, and the result was checked by sequencing of the PCR product. In addition, molecular mechanisms regulating flower colour in the Oriental lily hybrid cultivars 'Perth' and 'Gran Tourismo' were also investigated and discussed.

2. Materials and methods

2.1. Plant materials

Four lily cultivars including L. *longiflorum* 'Lincoln' (white tepals), Oriental hybrid lily cultivars 'Rialto' (white tepals), 'Perth' (pink tepals) and 'Gran Tourismo' (red tepals) were used (see Fig. 2, stage 5 for flower colours). The bulbs were purchased from De Jong Lelies Holland BV (Andijk, The Netherlands) and were stored at −1 °C and briefly transferred to 4 °C for 24 h before planting. The bulbs were planted in crates (30 cm × 50 cm, 6 bulbs per crate) filled with commercial jiffy substrate (Jiffy Products International B.V., Moerdijk, The Netherlands). The plants were grown in a greenhouse at 21 °C during the day and 19 °C during the night (16/8 h photoperiod) with relative humidity at 70% until anthesis.

Flowers were divided into five developmental stages starting from the bud until a fully open flower. Stages were determined on the basis of colour formation on the bud surfaces. Stage 1 (2–5 cm) buds contained no anthocyanin pigmentation, stage 2 (5.1–8 cm) buds began pigmentation at the basal part, stage 3 (8.1–10 cm) bud surfaces were lightly coloured, stage 4 buds (>10 cm) showed full colouration on bud surfaces, and stage 5 was fully open flowers (Fig. 2). Buds or flowers with detached stamens and pistils were harvested for anthocyanin measurement, RNA and gDNA isolation. The inner and outer tepals were combined together, immediately frozen in liquid nitrogen and kept in a −80 °C freezer until use.

2.2. Determination of anthocyanin contents

Anthocyanin and related compounds were extracted from tepals (100–200 mg dry weight) with 5–9 mL of extraction solvent containing 1% (*v*/v) hydrochloric acid (HCl) and 0.1% (v/v) butyl hydroxyl anisol

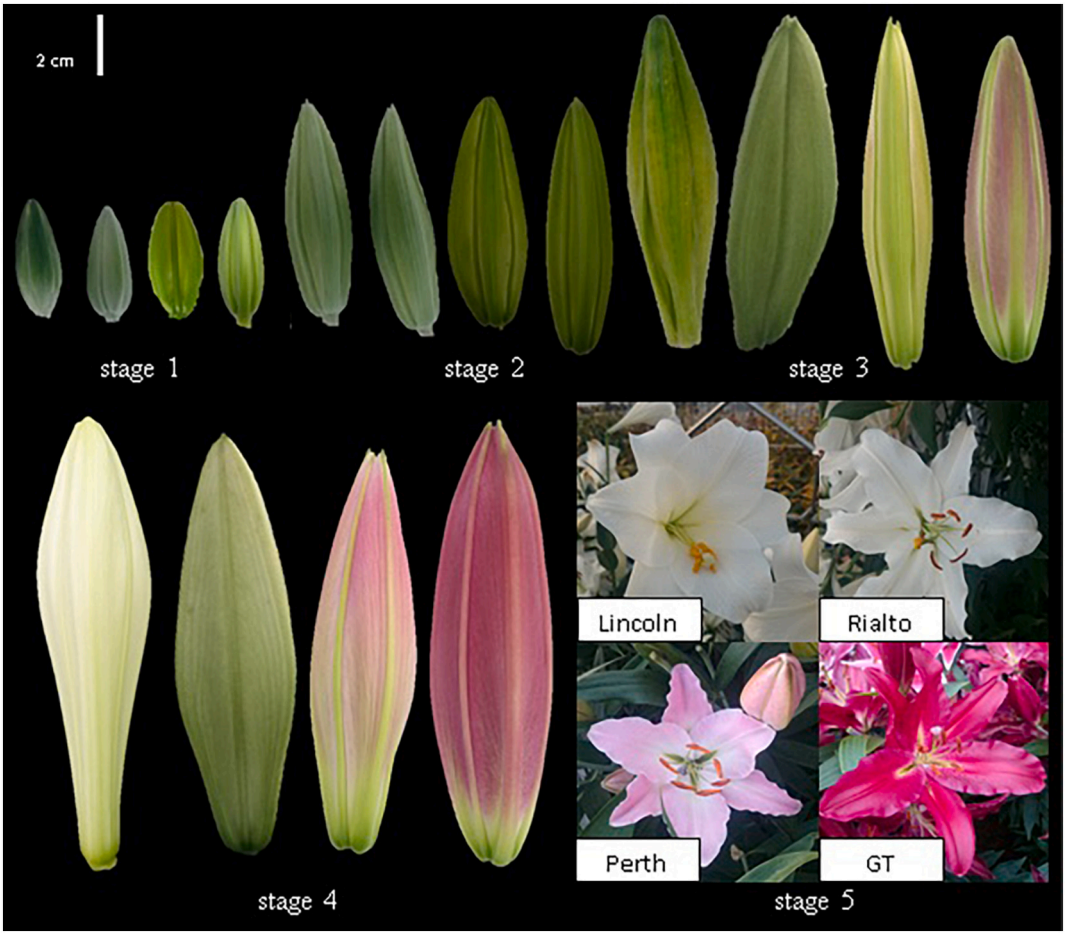

Fig. 2. Five flower developmental stages in *Lilium* cultivars used in this study. Flower buds from left: 'Lincoln'; 'Rialto'; 'Perth'; Gt, 'Gran Tourismo'.

(BHA) in methanol. Three replicates of each cultivar in each stage were used for extraction. The extraction mixture was sonicated for 15 min in VWR ultrasonic cleaner (International bvba/sprl, Leuven, Belgium) followed by shaking at 500 rpm in a shaker type HLC for 15 min at 20 °C, centrifugation in a Heraeus Multifuge 3S (Kendo Laboratory Products, Germany) for 10 min at 4200 rpm, and the supernatant was collected. This procedure was repeated at least three times or until all the anthocyanins were removed. Acid hydrolysis was performed in order to prepare the anthocyanidin aglycones; a mixture of 0.5 mL of sample solution with 0.5 mL of 2 N HCl was heated in a heating block set for 120 min at 99 °C.

The hydrolysed samples (Fig. 3A) were analysed by high performance liquid chromatography (HPLC), using a Waters Alliance e2695 system with a Waters 2996 photodiode array detector (Milford, MA, USA) and a HyPURITY C18 column (3 μm particle size, 150 mm × 3 mm, Thermo Scientific, Cheshire, UK) at 40 °C, the flow rate of 0.8 mL/min, and aliquots of 10 μL were injected. Absorption spectra were monitored at 250–550 nm. Eluent A was 0.1% (*v*/v) trifluoroacetic acid in MilliQ water and eluent B was 0.1% (v/v) trifluoroacetic acid in acetonitrile. A linear gradient elution as follows was performed: 5–28% of eluent B at 0–35 min, 28–75% of eluent B at 35–37 min, 75% of eluent B at 37–40 min, 75–5% of eluent B at 40–42 min, 5% of eluent B at 42–50 min. Chromatograms were acquired at 512 nm (anthocyanins), 365 nm (quercetin), and 280 nm (dihydrokaempferol and dihydroquercetin or taxifolin). Six concentrations (0.005, 0.01, 0.025, 0.05, 0.1 and 0.2 mg mL^{-1}) of cyanidin -, pelargonidin -, delphinidin chloride, dihydrokaempferol, dihydroquercetin (taxifolin) and quercetin were used to make calibration curves. Quantification of anthocyanins and related compounds was performed by correlating the chromatographic peak area with concentrations in accordance with the calibration curve of the corresponding external standard.

2.3. gDNA isolation, PCR amplification and sequencing

To determine the presence or absence of the target genes in the plant genome, total gDNA was isolated from tepals of stage 4 buds of each cultivar using DNeasy® Plant Mini Kit (250) Cat. No. 69106 according to the manufacturer's instructions. The final PCR reaction mixture (20 μL) contained 2 μL of each sample's gDNA as a template, 2 μL of 2.5 mM 10× PCR buffer, 0.4 μL of 400 μM dNTP, 0.1 μL of 0.05 U/μl Dreamtaq polymerase, 1 μL of 10 μM each forward and reverse primers and 13.5 μL of miliQ water. *CHSb*, *CHIa* and *ANS* were amplified using primers from [15]. To amplify *F3H*, *DFR*, *MYB12*, *MYB15* and *bHLH2*, gene specific primers were designed based on multiple alignments of *Lilium* sequences available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). They are presented in Table S1. The amplified fragments were sequenced. Multiple sequence alignments were performed with homologous sequences from other *Lilium* using SeqMan Pro (DNAstar, Inc.). The coding sequences were translated into amino acid sequences using a translator (http://fr33.net/translator.php), and then used for a Blastp search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify genes showing high similarities with the amino acid sequences. The amino acid sequences were aligned using ClustalΩ (Omega) with default parameters (Sievers and Higgins, 2014).

2.4. RNA isolation and cDNA synthesis

Total RNA was isolated from tepals (100 mg fresh weight) using the

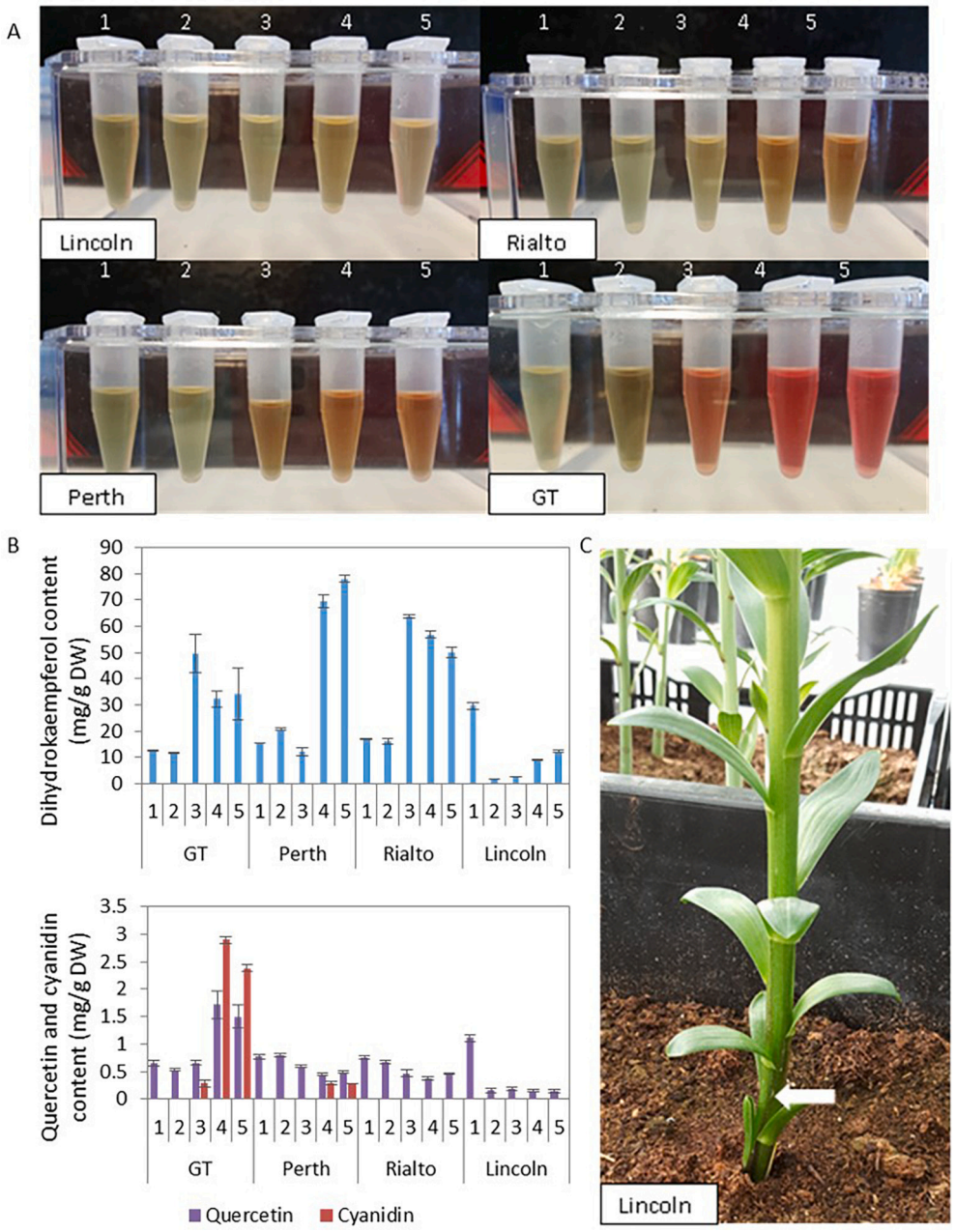

Fig. 3. A. Acid hydrolysed extracts used for HPLC analysis. B. Anthocyanin contents in tepals of four *Lilium* cultivars during five flower developmental stages. Vertical bars indicate the standard error of three replicates. C. 'Lincoln' plant showing a streak of anthocyanin pigmentation at basal stem, marked with white arrow. Gt, 'Gran Tourismo'.

RNeasy mini kit from Qiagen (Hilden, Germany) following the manufacturer's instruction. The quantity and quality of RNA were determined using Nanodrop1000™ and gel electrophoresis. RNA was treated with DNaseI (Invitrogen Thermo Fisher Scientific). Reaction mixture containing 1 μg RNA, 1 μL 10× DnaseI reaction buffer, 1 μL DnaseI and miliQ water adjusted to a total volume of 10 μL was incubated at 20 °C for 15 min. 1 μL 25 mM EDTA was added to the reaction mixture and further incubated at 65 °C for 10 min to stop the reaction and the mixture was held at 10 °C. First-strand cDNA was synthesized using iScript™ cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. In short, the total reaction mixture (20 μL) consisting of 11 μL Dnase treated RNA, 4 μL 5× iScript reaction mixture, 1 μL iScript reverse transcriptase and 4 μL Rnase free water was incubated in a PCR machine at 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, 4 °C for 5 min, 85 °C for 5 min and held at 10 °C. The cDNA was diluted ten times in miliQ water and kept at 4 °C until further use.

2.5. Quantitative real-time PCR (qPCR)

To investigate the transcription levels of anthocyanin structural genes (*CHSa*, *CHSb*, *CHIa*, *CHIb*, *F3H*, *F3'H*, *DFR* and *ANS*) and transcription factors genes (*MYB12*, *MYB15* and *bHLH2*) in four lily cultivars at five flower stages, quantitative real-time PCR (qPCR) was performed using CFX96TM real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA). The final reaction volume (10 μL) contained 2 μL of diluted first-strand cDNA, 5 μL 2*iQ SYBR GREEN super mix 2* DNA fluorescent dye (Bio-Rad Laboratories, Inc., Hercules, CA, #172–5006 CUST), 0.3 μL of each forward and reverse primers (10 μM), and 2.4 μL miliQ water. Cycling conditions were: preheating at 95 °C for 3 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by 95 °C for 10 min

and 65 °C to 95 °C (0.5 °C increment) for 5 s. The specific qPCR primers used in this study were designed based on nucleotide sequences from other *Lilium* species available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and some were extracted from literature (Table S2). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was selected to normalize the differences in the quantity of mRNA of target genes. Additionally, efficiency for each primer set used in this study was calculated using LinRegPCR software (Ruijter et al., 2009), and is given in Table S2. The relative gene expression (RGE) was determined based on the $2^{-\delta Ct}$ calculation method, where δCt = Ct (target gene) – Ct (GAPDH) (Livak and Schmittgen, 2001). Three biological replicates of each cultivar in each stage were used in the analysis.

2.6. Statistical analysis

Data was subjected to one-way analysis of variance (ANOVA) and means were compared using Fisher's Least Significant Difference (LSD) test at $P < 0.05$. To determine correlations between gene expression involved in anthocyanin formation in the Oriental lily cultivars 'Gran Tourismo' and 'Perth', gene expression of two cultivars were combined into a single dataset, then Spearman correlation coefficient was analysed using GenStat 18th edition.

3. Results

3.1. Anthocyanin accumulation in tepals

Accumulation of cyanidin (anthocyanin), dihydrokaempferol (dihydroflavonol) and quercetin (flavonol) was quantified in lily tepals on the basis of their retention time and absorption spectra by HPLC analysis (Fig. 3B). Cyanidin accumulated in tepals of the coloured cultivars 'Perth' and 'Gran Tourismo', whilst no cyanidin was detected in the white-flowered cultivars 'Rialto' and 'Lincoln'. The accumulation of cyanidin in 'Gran Tourismo' began at stage 3 bud, peaked at stage 4 bud, and then slightly dropped at stage 5 open flower. Cyanidin accumulated in 'Perth' at stages 4 and 5, but the quantity was significantly lower (approximately 2-fold) compared to that of 'Gran Tourismo'. These results showed that pigmentation began at different flower developmental stages in different cultivars and colour variation in tepals was related to anthocyanin accumulation.

Lily tepals accumulated much higher amounts of dihydrokaempferol compared to quercetin. Although the average amount of dihydrokaempferol in 'Lincoln' was much lower than to that of three Oriental lily cultivars, the overall results gave an indication for the functionality in all cultivars of the early structural genes (*LlLinCHSa*, *LlLinCHSb*, *LlLinCHIa*, *LlLinCHIb*, *LlLinF3H*) in the pathway and of the transcription factors (*LlLinMYB12* and *LlLinMYB15*), which are thought to activate the transcription of the early structural genes. Quercetin was detected in all four cultivars. In 'Lincoln', dihydrokaempferol and quercetin were predominantly present at stage 1, which fits if dihydrokaemperol was a precursor for quercetin requiring both *LlLinF3'H* and *LlLinFLS* activities. However, the later rise of dihydrokaempferol, for example at stage 5, did not lead to a higher level of quercetin. Nevertheless, 'Lincoln' contained a significantly lower amount of quercetin compared to that of 'Rialto', 'Perth' and 'Gran Tourismo'. These results indicated that the activity of *LhFLS* must be rather high in those three Oriental cultivars compared to 'Lincoln'. Dihydroquercetin was detected but the level was below the quantification level in all four cultivars.

3.2. Genes presence/absence

The presence/absence of target genes in tepals was demonstrated based on a series of gene-specific PCR amplifications performed on gDNA as template. Identity of the amplified fragments were confirmed by sequencing the PCR products and their deduced amino acid sequences were checked by Blastp search (Table S3). Amplified fragments suggested that all three Oriental lily cultivars 'Rialto', 'Perth' and 'Gran Tourismo' contained all eight structural genes (*LhCHSa*, *LhCHSb*, *LhCHIa*, *LhCHIb*, *LhF3H*, *LhF3'H*, *LhDFR*, *LhANS*) and three transcription factors (*LhMYB12*, *LhMYB15*, *LhbHLH2*). In accordance with the observed lack of expression, we failed to amplify *LlLinF3'H*, *LlLinDFR* and *LlLinbHLH2* from L. *longiflorum* cultivar 'Lincoln' and no amplicon of these genes could be obtained even when varying PCR reaction conditions and using various primers (data not shown). This result indicated a genetic block in later biosynthetic steps that could responsible for the absence of anthocyanin accumulation in the white tepals of 'Lincoln'. On the other hand, the *LhRiaDFR* gene was found to be present in 'Rialto', however not expressed.

3.3. Sequences of DFR, ANS and MYB12

Given the presence of *LhRiaDFR* but no expression in 'Rialto' as mentioned above, we hypothesized that there is a defect in this gene that can impair its function and/or a mutation in the *LhRiaMYB12* transcription factor that weakened the expression of anthocyanin structural genes (*LhRiaDFR* and *LhRiaANS*). To test these hypotheses, full-length or partial amino acid sequences of *DFR*, *ANS* and *MYB12* from *Lilium* cultivars were deduced and analysed.

Full-length *LhGtDFR* and *LhPerDFR* sequences respectively from 'Gran Tourismo' and 'Perth' together with a partial sequence *LhRiaDFR* from 'Rialto' were compared with *LsDFR* sequences from L. *speciosum* (Fig. 4). *LsDFR*-rr1 from the red tepals/red anthers line of L. *speciosum* differed from *LhGtDFR* and *LhPerDFR* by three unique amino acid changes (V to G, M to V and M to I). The *LhGtDFR* sequence was similar to that of *LhPerDFR*, except for two amino acid changes from isoleucine (I) to threonine (T) and glutamine (Q) to glutamic acid (E). The *LhRiaDFR* sequence resembled *LsDFR*-wr from the white tepals/red anthers line of *L. speciosum* which was also identical to *LsDFR*-rr2 from the red tepals/red anthers line. However, *LhRiaDFR* was unique compared to the other sequences by having four unique amino acid changes (S to P, C to S, N to D/H and Q to R). Nevertheless, no critical mutation such as a premature stop codon was found in the *LhRiaDFR* partial sequence.

For *ANS*, a full-length sequence from the three Oriental hybrid lily cultivars 'Gran Tourismo', 'Perth, 'Rialto' and a partial sequence from *L. longiflorum* cultivar 'Lincoln' were identified and compared (Fig. 5). *LhGtANS* differed from the ones in *LhPerANS* and *LhRiaANS* only by two amino acid changes from valine (V) to glycine (G) and glutamine (Q) to histidine (H). However, the sequence of *LlLinANS* was not determined at the positions where the two changes in the other cultivars were detected.

A full-length sequence of *LhGtMYB12*, *LhPerMYB12*, *LhRiaMYB12* and a partial sequence of *LlLinMYB12* were identified and compared (Fig. 6). The *LlLinMYB12* sequence was outside of the R2 and R3 repeats, toward the C-terminus, and contained five unique amino acid changes. The *LhGtMYB12* contained eight unique changes compared to that of *LhPerMYB12* and *LhRiaMYB12*. However, *LhPerMYB12* sequence was identical to *LhRiaMYB12*, and both contained leucine (L) instead of tryptophan (W) in one of the highly conserved W residues in the R2 repeat.

3.4. Expression of anthocyanin biosynthesis-related genes

Relative gene expression of eight structural genes (*CHSa*, *CHSb*, *CHIa*, *CHIb*, *F3H*, *F3'H*, *DFR*, *ANS*) and three transcription factor genes (*MYB12*, *MYB15*, *bHLH2*) in tepals of four *Lilium* cultivars were analysed during five flower development stages (Fig. 7). The RGE was categorized into four categories; hardly expressed ($0 < RGE < 0.1$), lowly expressed ($0.1 < RGE < 0.5$), moderately expressed ($0.5 < RGE < 2$) and highly expressed ($RGE > 2$). The transcription of the early structural genes (*CHSa*, *CHSb*, *CHIa*, *CHIb* and *F3H*) was detected in tepals of all four cultivars. The genes were highly expressed in 'Gran Tourismo', and were transcribed during all flower developmental stages, but mostly peaked at stage 4 bud. In 'Perth', those early structural genes were lowly

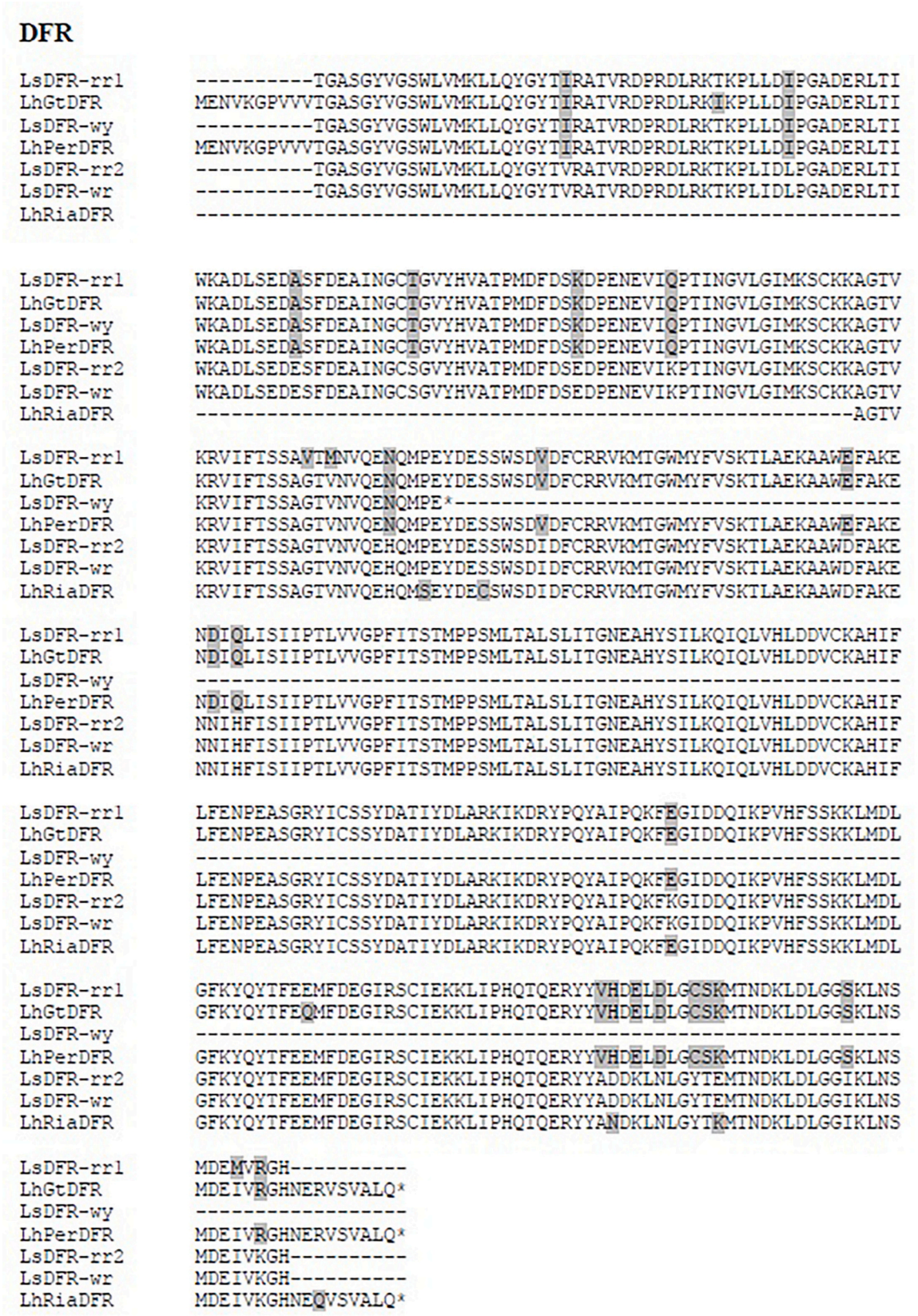

Fig. 4. Multiple sequence alignment of *Lilium DFR*. Letters on grey background indicate amino acid substitutions. Stop codon is marked with an asterisk (*). Lh, *L. hybrida*; Ll, *L. longiflorum*; Gt, 'Gran Tourismo'; Per, 'Perth'; Ria, 'Rialto'; Lin, 'Lincoln'; Ls, *L. speciosum*; rr, red/red line, wy, white/yellow line, wr, white/red line of L. *speciosum*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

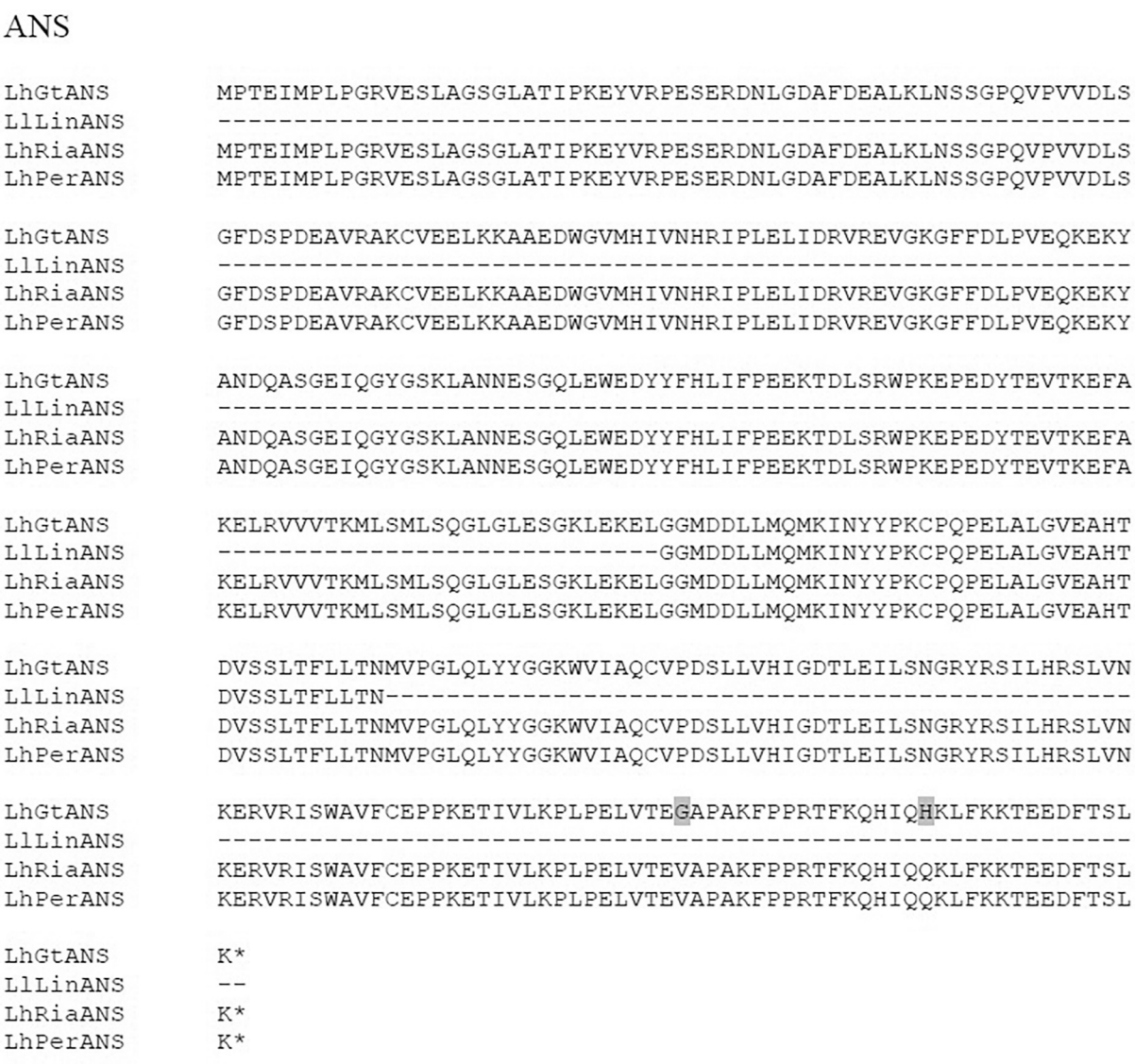

Fig. 5. Multiple sequence alignment of *Lilium ANS*. Letters on grey background indicate amino acid substitutions. Stop codon is marked with an asterisk (*). Lh, *L. hybrida*; Ll, *L. longiflorum*; Gt, 'Gran Tourismo'; Per, 'Perth'; Ria, 'Rialto'; Lin, 'Lincoln'.

expressed, except *LhPerCHSb,* which was hardly expressed. 'Rialto' had moderately expressed *LhRiaCHIa*, but hardly to lowly expressed *LhRiaCHSa*, *LhRiaCHSb*, *LhRiaCHIb* and *LhRiaF3H*. In 'Lincoln', those early structural genes were lowly expressed, and *LlLinCHSb* was hardly expressed. The expression of early structural gene *F3'H* was generally low in all three Oriental cultivars. 'Gran Tourismo' stage 5 open flower had a higher expression level of *LhGtF3'H* than 'Perth' *LhPerF3'H* stage 5 open flower. Nevertheless, no expression of *LlLinF3'H* was detected in 'Lincoln'.

The late structural gene *LhGtDFR* was moderately expressed but *LhPerDFR* was lowly expressed. The expression level increased over the flowering stages up to stage 4 and then slowly decreased at stage 5 in both coloured cultivars. However, no expression of *LhRiaDFR* and *LlLinDFR* was observed. *LhRiaANS* and *LlLinANS* were hardly expressed in white tepals of 'Rialto' and 'Lincoln' respectively. Also, *LhGtANS* was lowly expressed in 'Gran Tourismo' and *LhPerANS* was hardly expressed in 'Perth'.

The expression levels of transcription factors (*MYB12*, *MYB15* and *bHLH2*) were as follows. In 'Gran Tourismo', *LhGtMYB12* was hardly expressed in stages 1 and 2 buds, upregulated at stage 3 bud, peaked at stage 4 bud, and then dropped at stage 5 open flower. A similar pattern was observed in 'Perth' and 'Rialto', but the levels were much lower than that of 'Gran Tourismo'. The white-flowered cultivar 'Lincoln' had hardly expressed *LlLinMYB12* in tepals. Another *MYB* transcription factor, *MYB15* was hardly expressed in all four cultivars with 'Gran Tourismo' showing the highest expression level. The 'Gran Tourismo' *LhGtMYB12* and *LhGtMYB15* showed an expression pattern similar to that of *LhGtCHSa*, *LhGtCHSb*, *LhGtCHIa*, *LhGtF3H* and *LhGtDFR* transcription profiles. In contrast, a different expression pattern was observed in *LhbHLH2*. The transcription of *LhPerbHLH2* and *LhRiabHLH2* decreased over the flowering stages in 'Perth' and 'Rialto' respectively. In 'Gran Tourismo', *LhGtbHLH2* was hardly expressed and no expression of *LlLinbHLH2* was seen in 'Lincoln'.

3.5. Regulation pattern of transcription factors

The gene expression data showed that many structural genes display similar expression patterns as *LhMYB12* and *LhMYB15*, suggesting some co-regulation. However, *LhbHLH2* seemed to act in an opposite way. In order to understand the co-expression pattern in gene expression data, a correlation analysis between expression levels of genes in the anthocyanin pathway in 'Gran Tourismo' and 'Perth' was performed (Fig. 8). The expression of all structural genes were strongly correlated with each other (all rho above 0.90), with the exception of *LhF3'H*, which has

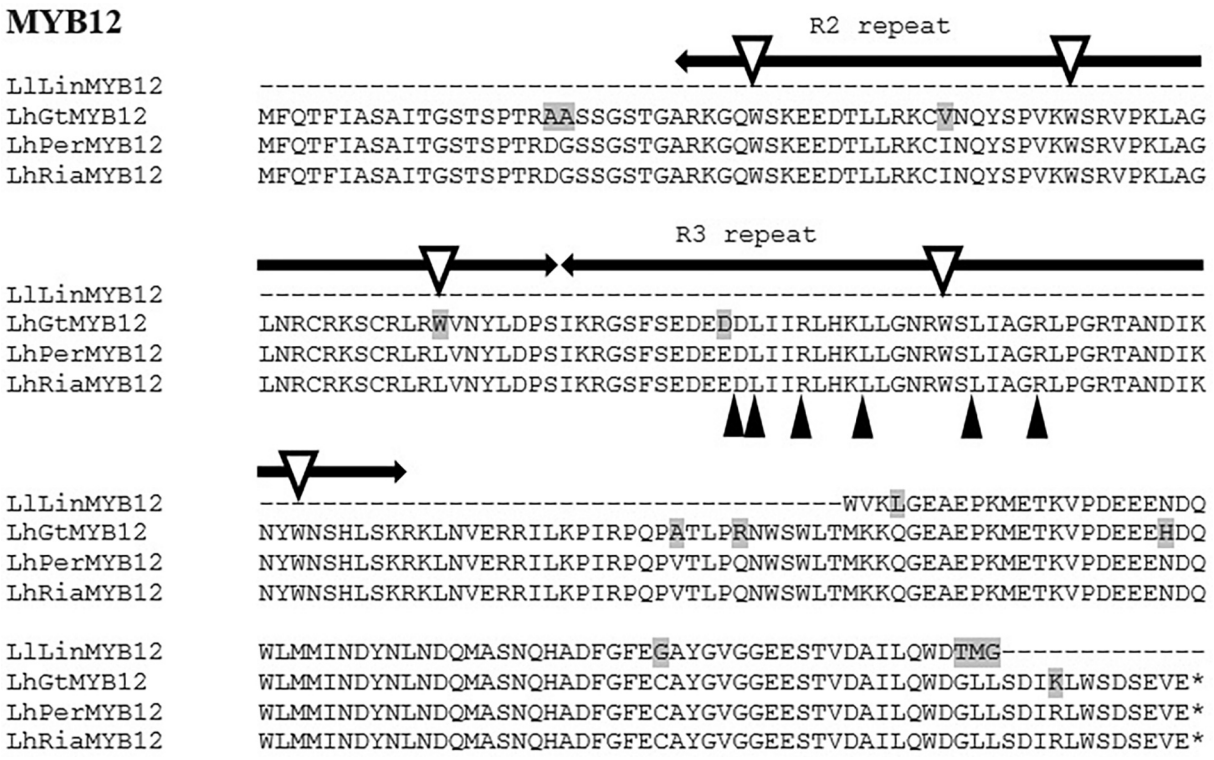

Fig. 6. Multiple sequence alignment of *Lilium MYB12*. Letters on grey background indicate amino acid substitutions. Stop codon is marked with an asterisk (*). White arrowheads indicate the conserved W residues in the R2 and R3 repeats. Black arrowheads indicate the motif [D/E]Lx2[K/R]x3Lx6Lx3R in the R3 repeat required for interactions with *bHLH* transcription factor. Lh, *L. hybrida*; Ll, *L. longiflorum*; Gt, 'Gran Tourismo'; Per, 'Perth'; Ria, 'Rialto'; Lin, 'Lincoln'.

weaker correlation coefficient (rho = 0.34 to 0.56). The transcription factor, *LhMYB12* showed a positive correlation with all the structural genes (rho = 0.72 to 0.90), including *LhF3'H* (rho = 0.32). *LhMYB15* was also positively correlated with all the structural genes although the correlation coefficient was generally weaker than that of *LhMYB12*. However, all the structural genes and the *MYB*s were negatively correlated with *bHLH2*. The correlation analysis suggested that *LhMYB12* and *LhMYB15* play an important role in regulating the anthocyanin structural genes in tepals of 'Gran Tourismo' and 'Perth'. In contrast, *LhbHLH2* seemed not essential in regulating the expression of the structural genes.

4. Discussion

For an improvement of anthocyanin engineering strategies, a careful characterisation of the structural genes and transcription factors involved in a biosynthetic pathway is very important. Therefore, understanding the genetic background behind white colouration in *Lilium* sp. is necessary. In this study, we determined the molecular mechanisms responsible for white flower colour development in the L. *longiflorum* cultivar 'Lincoln' and in the Oriental lily hybrid cultivar 'Rialto', and compared them to the coloured cultivars 'Perth' and 'Gran Tourismo'. We found that several mechanisms were responsible for white colouration in tepals of 'Lincoln' and 'Rialto'. In 'Lincoln', we were unable to amplify genomic sequences of two structural genes (*LlLinF3'H* and *LlLinDFR*) and one transcription factor (*LlLinbHLH2*), resulting in no transcripts of the genes that could be detected by qPCR. In 'Rialto', *LhRiaDFR* was present, but it was not transcribed. No premature stop codon that can impair *LhRiaDFR* protein function was found in 'Rialto', as was determined in the white tepals/yellow anthers line of L. *speciosum* (Suzuki et al., 2015). *LhRiaANS* and *LlLinANS* were hardly expressed in both white-flowered 'Rialto' and 'Lincoln' respectively. However, *LhRiaANS* amino acid sequence resembled the *LhPerANS* of 'Perth' sequence, indicating no amino acid mutation in *LhRiaANS* of the white tepal of 'Rialto'. All of the analysed genes and transcription factors are present and transcribed in the tepals of the coloured cultivars 'Perth' and 'Gran Tourismo'. Some amino acid changes occurred, but no major defects that could impair protein structure were observed.

In many *Lilium* species, *MYB12* usually interacts co-ordinately with *bHLH2* forming a *MYB12/bHLH2* complex to upregulate the transcription of structural genes (Lai et al., 2012; Suzuki et al., 2015; Nakatsuka et al., 2009; Yamagishi, 2011; Yamagishi et al., 2012). In the white tepals of 'Rialto' and L. *speciosum*, the W-to-L substitution in the R2 repeat of *MYB12* was reported to be responsible for the absence of transcriptional activation of anthocyanin structural genes causing the lack of anthocyanin accumulation (Suzuki et al., 2015). We also found this W-to-L substitution in *LhPerMYB12* (Fig. 6). This observation questions the suggested effect of W-to-L amino acid substitution in the R2 repeat. Although the highly conserved W residues are thought to be essential for the DNA binding domain (Ogata et al., 1992) and are required to maintain the function of *R2R3-MYB* (Yamagishi, 2011), our data suggested that the function of *LhRiaMYB12* and *LhPerMYB12* containing a W-to-L amino acid substitution in the R2 repeat seems to be maintained. Firstly, because 'Perth' has pink tepals. Secondly, because the structural genes *LhPerDFR* and *LhPerANS,* which are supposed to be regulated by *LhPerMYB12,* were transcribed in 'Perth' tepals; this signifies the functionality of the *LhPerMYB12* gene. Thirdly, 'Rialto' has red anthers (see Fig. 2, stage 5), which also suggests the functionality of the anthocyanin biosynthetic pathway. Nevertheless, we cannot reject the possibility that the W-to-L substitution reduces anthocyanin accumulation in tepals through a weakened interaction with other transcription factors such as bHLH or WD-repeat. In line with this was our observation that the expression levels of *LhPerMYB12* and *LhRiaMYB12* were relatively low compared to *LhGtMYB12* from 'Gran Tourismo'.

In 'Gran Tourismo', *LhGtMYB12* having the conserved W residues, was hardly expressed in stages 1 and 2 buds, upregulated at stage 3 bud, peaked at stage 4 bud, and slightly dropped at stage 5, open flower. The expression patterns of the newly discovered *LrMYB15* from L. *regale*

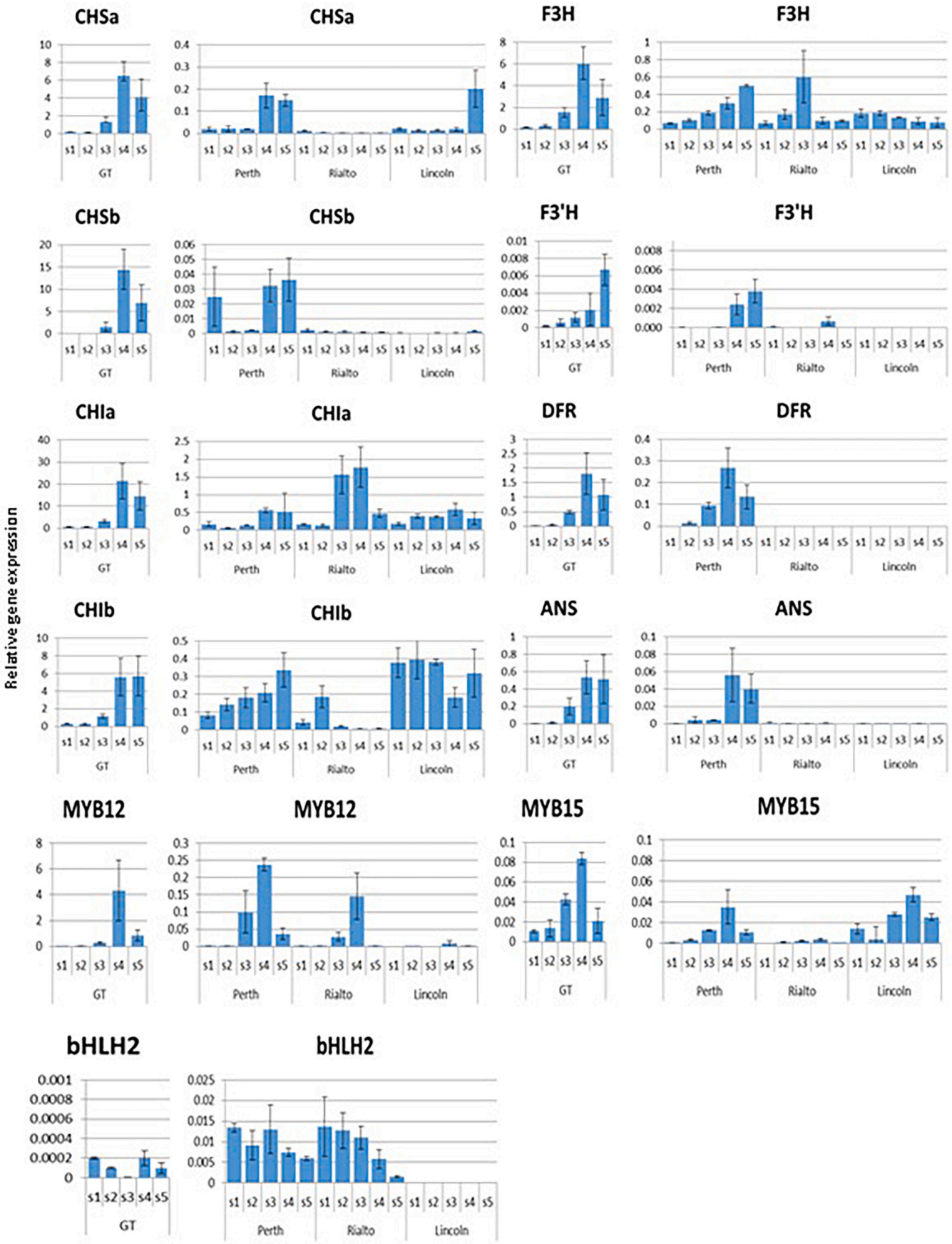

Fig. 7. Relative levels of gene expression of eight structural genes and three transcription factors during five flower developmental stages in Oriental hybrid lily cultivars 'Gran Tourismo', 'Perth', 'Rialto' and L. *longiflorum* cultivar 'Lincoln'. Vertical bars indicate the standard error of three biological replicates.

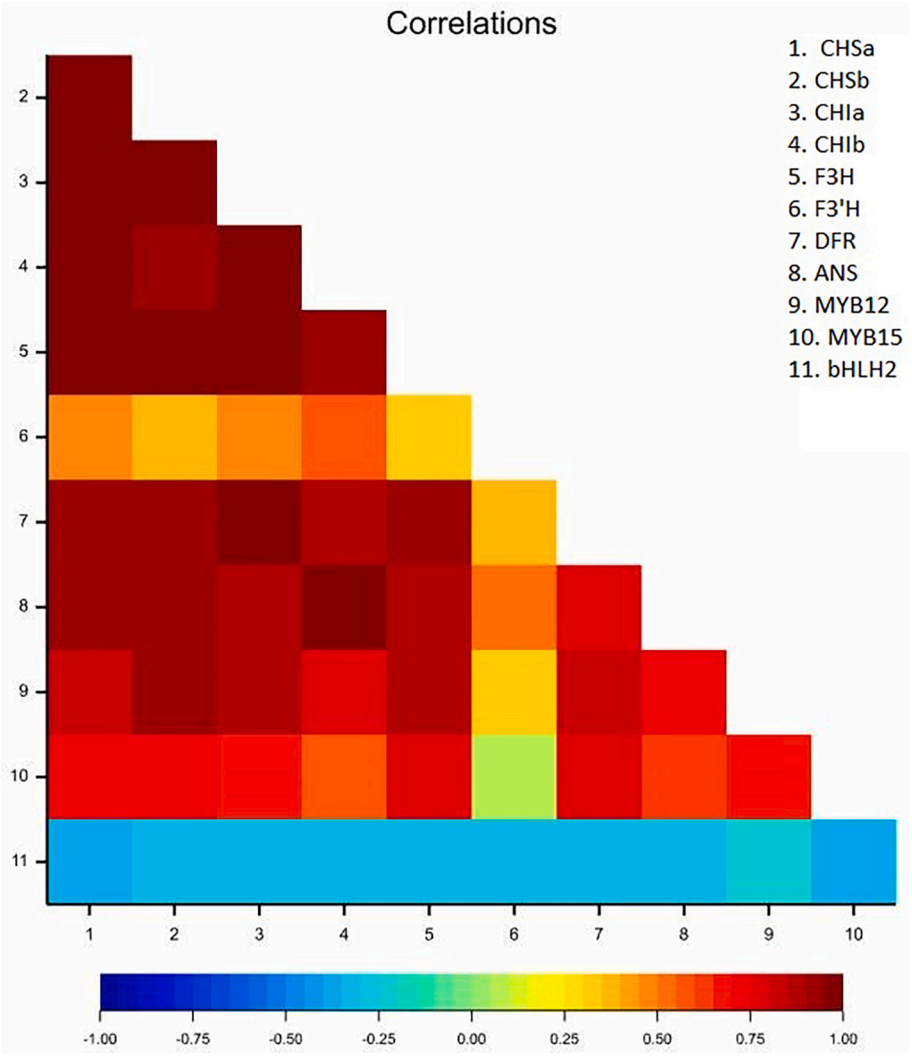

Fig. 8. Correlations between gene expressions involve in colouration in lily cultivars 'Gran Tourismo' and 'Perth'. Colour intensity represents Spearman rank correlation coefficient (*rho*) value.

(Yamagishi, 2016) and *LhMYB12* from the Oriental cultivars were similar, although the expression levels of *LrMYB15* were very much lower compared to *LhMYB12*. Anthocyanin structural genes showed a similar expression pattern as the *MYB*s in the coloured cultivars 'Gran Tourismo' and 'Perth', and they were positively correlated. On the other hand, the expression profile of the other supposedly important transcription factor, *LhbHLH2,* showed a negative correlation to those of the structural genes in both coloured cultivars, and the transcription level of *LhGtbHLH2* was extremely low in the red tepals of 'Gran Tourismo'. This suggested that in 'Gran Tourismo', *LhGtbHLH2* is not essential in inducing the expression of structural genes in the tepals but that this is primarily due to the control of *LhGtMYB12* and *LhGtMYB15*, and perhaps only a few transcripts of *LhGtbHLH2* are required as a co-factor to support the function of the *R2R3-MYB*. It was demonstrated in *N. benthamiana* that introduction and expression of *LrbHLH2* from *L. regale* did not stimulate the transcription of *LrDFR* and *LrANS* (Yamagishi, 2016). However, expression of *LrbHLH2* together with *LrMYB15* enhanced the transcription of *LrDFR* and *LrANS*, and the levels were higher than that induced by *LrMYB15* alone. The key role of *R2R3-MYB* transcription factors in inducing the transcription of anthocyanin structural genes has been reported in several other plant species such as *Arabidopsis* (Borevitz et al., 2000), snapdragon (Schwinn et al., 2006), petunia (Quattrocchio et al., 1999), strawberry (Kortstee et al., 2011), and litchi (Lai et al., 2014). Another explanation could be the presence of other *bHLH*s transcription factors that are not yet characterized in *Lilium* sp.

Transcription profiles of eight structural genes (*CHSa*, *CHSb*, *CHIa*, *CHIb*, *F3H*, *F3'H*, *DFR* and *ANS*) and metabolite accumulation were examined during flower development. 'Gran Tourismo' and 'Perth' accumulated a single anthocyanin, i.e. cyanidin, in tepals. The white tepals of 'Rialto' and 'Lincoln' did not contain anthocyanin. High transcript accumulation was observed in the red tepals of 'Gran Tourismo', followed by the pink tepals of 'Perth'. The white tepals of 'Rialto' and 'Lincoln' showed the least transcript accumulation. During flower development of 'Gran Tourismo', no or low anthocyanin structural gene expression was detected at the early stages 1 and 2 buds, respectively. The expression increased at stage 3 bud, peaked at stage 4 bud, and slightly dropped at stage 5 open flower. These expression profiles were correlated with the anthocyanin accumulation profiles. The expression level of *LhGtF3'H* was very low compared to the other genes. Similar results have been reported in the Asiatic hybrid lily cultivar 'Lollypop' which has bicolour tepals with pigmented tips and white bases (Suzuki et al., 2016). 'Gran Tourismo' did not contain measurable levels of dihydroquercetin (DHQ) in tepals, which might be explained by the low accumulation of *LhGtF3'H* transcripts. The transcription of *LhPerF3'H* and *LhRiaF3'H* was even lower, and *LlLinF3'H* could not be determined in 'Lincoln'. As 'Gran Tourismo' did contain cyanidin, *LhGtF3'H* enzyme activity must be present, despite low gene expression. The absence of

measurable dihydroquercetin suggested a very active *DFR* enzyme and rapid conversion of DHQ into leucocyanidin. According to Lai et al. (2012), Asiatic hybrid lily cultivars including white-flowered cultivars do not accumulate flavonols or flavones in tepals, except for cultivar 'Landini', where small amounts of flavonols (quercetin derivatives) accumulated in tepals. In the present study, we found high accumulation of dihydrokaempferol in tepals of 'Gran Tourismo', 'Perth', 'Rialto' and 'Lincoln'. This result indicated the activity and functionality of early structural genes (*CHSa*, *CHSb*, *CHIa*, *CHIb*, *F3H*) in the pathway in all four cultivars.

DFR, a key enzyme involved in anthocyanin production in plants, e.g. in grape (Gollop et al., 2002) and sweet potato (Wang et al., 2013), was expressed in the coloured tepals of Oriental lily hybrid cultivars 'Gran Tourismo' and 'Perth'. However, the *LhGtDFR* and *LhPerDFR* sequences only differed by two amino acid substitutions. The *DFR* expression profiles differed from cultivar 'Sorbonne', as in this cultivar, a steep decrease between stage 4 and 5 was observed (Yamagishi, 2011), while in 'Gran Tourismo' and 'Perth', the decrease is much smaller. A similar small decrease in *DFR* expression between stages 4 and 5 was also seen in cultivar 'Montreux' (Yamagishi et al., 2010). Suzuki et al. (2015) found a premature stop codon in *LsDFR*-wy, and suggested this mutation caused the white colouration in tepals of L. *speciosum,* a white tepals/yellow anthers line. In 'Lincoln', however, no *LlLinDFR* genomic copy could be detected, and no *LlLinDFR* transcript was expected nor found, and consequently no anthocyanin production occurred in the white tepals. On the other hand, a colour change from colourless to reddish in the extract of L. *longiflorum* during acid hydrolysis was observed, which suggested the presence of *LlLinDFR* activity in this species (Martens et al., 2003). However, the colour change was not clear in our experiment (Fig. 3A). Based on the observation of a streak of anthocyanin pigment at the basal stem of L. *longiflorum* cultivar 'Lincoln' (Fig. 3C), we postulate that the anthocyanin pathway in 'Lincoln' may only be active in the very early stages of plant development, and is suppressed at the later stages and in flowers. That also means that the *LlLinDFR* gene should be present in this species but that we were unable to amplify it by PCR, which probably is due to the presence of multiple *DFR* gene copies with high variation in the sequences between *Lilium* spp. These observations suggested that genomic PCR does not offer a conclusive evidence for the absence of the gene. Therefore, an enzyme activity assay is recommended for future studies.

In this study, we found a *LhRiaDFR* genomic copy present in 'Rialto', but the gene was not expressed. Four unique amino acid substitutions were found in *LhRiaDFR* from 'Rialto' compared to other Oriental lilies and L. *speciosum* (Fig. 4). However, no critical mutation causing a premature stop codon was found, as reported in *LsDFR*-wy from the white tepals/yellow anthers line of L. *speciosum* (Suzuki et al., 2015). These results indicated no major defects that could impair *LhRiaDFR* protein function. Yet, it is possible that amino acid substitutions in *LhRiaDFR* decreased the binding affinity to dihydroquercetin. According to Johnson et al. (1999), dihydroquercetin differs from dihydrokaempferol and dihydromyricetin only by the number of hydroxyl groups on the B-ring. Thus, a *DFR* enzyme that has one or a few mutation points affecting a hydrogen bonding residue could show a dramatic decrease in the binding affinity to take up dihydroquercetin as a substrate.

ANS expression is generally low in all four lily cultivars. However, red tepals of 'Gran Tourismo' displayed a higher expression compared to the pink tepals of 'Perth'. In both white tepals, *LhRiaANS* and *LlLinANS* were hardly expressed. Similarly in white tepals of L. *speciosum*, hardly any expression of *LsANS* was detected (Suzuki et al., 2015). In addition, it was demonstrated that lowering *ThANS* expression yielded a paler petal colour in *Torenia hybrida* (Nakamura et al., 2006). Because *ANS* is involved in one of the final steps of the anthocyanin biosynthetic pathway, converting colourless leucoanthocyanidin into coloured anthocyanidin (Nakajima et al., 2006), expression of this gene is critically important in determining anthocyanin pigmentation.

5. Conclusion

In conclusion, 'Lincoln' is white possibly because of the inability to detect two structural genes (*LlLinF3'H* and *LlLinDFR*) and one transcription factor gene (*LlLinbHLH2*). No transcription of *LhRiaDFR* seemed the most probable cause of white tepals in 'Rialto'. All the analysed structural genes displayed much higher expression in the red tepals of 'Gran Tourismo' compared to the other cultivars. This result indicated that anthocyanin accumulation is highly correlated with the expression of the structural genes, which are regulated by *LhMYB12* and *LhMYB15*. In contrast, *LhbHLH2* did not seem to be essential in inducing the expression of structural genes, as it is hardly expressed in the red tepals of 'Gran Tourismo'. Our results suggest that developing cyanidin-based flower colour in 'Lincoln' might require expression of an *LlLinF3'H* gene that is able to catalyse dihydrokaempferol reduction, together with expression of *LlLinDFR* and *LlLinANS* genes that can efficiently catalyse dihydroquercetin reduction. 'Rialto' may require the introduction and active expression of *LhRiaDFR* and *LhRiaANS* genes. The RGE of the analysed genes was mostly highest in stage 4 buds, indicating that future transient gene transformation assays are best at this stage. These findings can pave the path for changing of white-flowered L. *longiflorum* and Oriental lily hybrid cultivars into flowers with novel colour.

Author contribution statement

HNNF and KW performed the experiments and analysed the data. JGS and MO helped with the experiments. FAK designed this research. HNNF wrote the manuscript. RGFV and FAK revised the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plgene.2022.100372.

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