Molecular determination and genetic modification of flower colour in *Lilium* spp.

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CHAPTER 1

General Introduction

***Lilium* spp.**

Lilies belong to the genus *Lilium* (family Liliaceae), consisting of more than 100 species that are widespread in the northern hemisphere, primarily in Asia, North America and Europe (Comber, 1949; De Jong, 1974; Lim et al., 2008). The species are taxonomically classified into seven sections based on various morphological and physiological characters. The seven sections are *Martagon, Pseudolirium, Lilium, Archelirion, Sinomartagon, Leucolirion,* and *Oxypetala* (Comber, 1949). Hybrids within sections *Archelirion, Sinomartagon* and *Leucolirion* represent the economically most important groups and are referred to as Oriental (O), Asiatic (A) and Longiflorum (L) hybrids (McRae, 1998). The major markets for commercial hybrids include fresh cut flowers, potted plants and garden plants. As cut flowers, lilies are ranked at number four in the Dutch list of economically most important ornamental crops with a total turnover amounted to 161 million Euro (Flora Holland, 2016).

Without a doubt, lilies are one of the most beautiful flowers and favoured by people around the world. They exhibit so many horticultural features such as flower colours, shapes and fragrances that everybody who likes flowers may have them as one of their favourites. The increasing demand for lilies has encouraged breeders to produce large numbers of hybrids and cultivars which combine as many of the desired traits as possible. Since 1960, more than 10,000 cultivars have been registered (Matthews, 2007). Among them, representatives of division I (Asiatic hybrids) derived from interspecific crosses between *Lilium* species belonging to the section *Sinomartagon*, and of division VII (Oriental hybrids) derived from interspecific crosses between *Lilium* species from the section *Archelirion* (Leslie, 1982), which are the most prominent ones (Lim and van Tuyl, 2006), and flower colour is their main feature. The flower colours of Asiatic hybrids vary from yellow, to orange, pink, red or white (Yamagishi, 2013). The Oriental hybrids have big, showy flowers in pink, red and white (Yamagishi, 2013, see also Fig. 1). On the other hand, representatives of division V (Longiflorum hybrids) derived from interspecific crosses between species belonging to the section *Leucolirion* only have white flowers (Fig. 1).

Fig. 1. *Lilium longiflorum* cultivar 'Lincoln' (upper left) and Oriental hybrid lily cultivars 'Rialto' (upper right), 'Perth' (bottom left) and 'Gran Tourismo' (bottom right) that were used in this study.

Breeding of commercial lilies

The breeding of commercial lilies can be traced back from the early 20th century when the Asiatic hybrids were bred. Asiatic hybrids are developed by crosses among *L. bulbiferum, L. davidii, L. maculatum* and *L. tigrinum* within the section *Sinomartagon,* whereas Oriental hybrids are developed by crosses among *L. auratum, L. japonicum, L. nobilissimum, L. rubellum* and *L. speciosum* within the section *Archelirion* (Leslie, 1982). The popularity of Asiatic hybrids started to decline after 1990 and was replaced by the growing interest in Oriental hybrids having larger-sized flowers and a strong fragrance (Younis et al., 2014). More than 300 Oriental cultivars such as 'Stargazer', 'Casa Blanca', 'Acapulco', 'Sorbonne', 'Crystal Light', 'Oberto', 'Siberia', 'Buckingham', and 'Conca d' Or' are popular in the market due to their outstanding flower colour, attractive shape and fragrance (Younis et al., 2014). Before World War II, the Japanese dominated the research and trade in lily bulbs, but after the Second World War, this was gradually taken over by researchers from the United

States reaching a peak in the 1970s. The leading cultivars at that time were 'Ace' and 'Nellie White', which were grown exclusively for flower decorations during Easter (http://www.liliumbreeding.eu/longi.htm). Then in the 1980s, two cultivars 'White American' and 'Snow Queen' were introduced in the Netherlands (Roh, 2011). *L. longiflorum* cultivars were gaining popularity among the growers and consumers because of certain positive traits such as outward-facing flowers, blooming in early spring, relatively easy to grow and a long vase life (Martens et al., 2003). However, they only exist in pure white-flowered cultivars, and thus varying their flower colours would be of potential commercial value.

Most of the commercial lilies nowadays are derived from classical breeding, in which normal hand pollinations are performed, seeds are germinated, and in the second (for Longiflorum), or third (for Asiatic), or fourth year (for Oriental) after crossing, seedlings are selected and propagated for evaluations (Lim and van Tuyl, 2006). Lily breeding for new cultivars is time-consuming. It takes 2 to 3 years from sowing of seed to first flowering (Nakano et al., 2005). Vegetative propagation to produce sufficient numbers of bulbs can take another 3 to 5 years. After the subsequent success of lily breeding in producing new interspecific hybrids like OA, LA, LO and others, several alternative breeding methods combining classical with modern (molecular) technologies have recently been developed and they are nicely reviewed by Younis and coworkers (2014).

All lily species are diploid (2n=2x=24), except a few triploid forms (2n=3x=36) of *L. tigrinum* and *L. bulbiferum* occurring in nature which are sterile (Noda, 1966; Noda and Schmitzer, 1990). Interspecific or intersectional hybridization involving crosses among species within or between sections (Lim et al., 2008) is the principle method in lily breeding, where success largely depends on the relationships between the chosen species (Krens and van Tuyl, 2011; Suzuki et al., 2016). Species that belong to the same section are easily crossable, and their F1 hybrids are fertile. They can be used for breeding because the ploidy of the hybrids can be maintained at diploid level. However, the species belonging to different sections are difficult to hybridize, and once obtained, the F1 hybrids are highly sterile (Karlov et al., 1999).

Wide interspecific crosses between *L. longiflorum* (L) and Asiatic (A) hybrid groups resulted mainly in sterile F1-hybrids (Karlov et al., 1999). This prevents further backcrossing, thus imposing a restriction to continue lily breeding along this line. The sterile hybrids are due to the failure in chromosome pairing during meiosis, which can be overcome by somatic chromosome doubling using certain chemicals such as colchicine and oryzalin (van Tuyl et al., 1992). However, such chemical treatments produced 'permanent hybrids', hybrids with identical gametes, with no pairing and recombination between parental genomes, and in this way the treatment provides little or no genetic variation among the progenies. As an alternative, the use of 2n gametes is a more preferred method in term of genetic variability (Karlov et al., 1999; Barba-Gonzalez et al., 2004). Induction of 2n gametes is quite possible

by application of nitrous oxide (N_2O) gas (Barba-Gonzalez et al., 2006). The advantage of this method is that it produces polyploids with recombination occurring between the parental genomes. Karlov et al. (1999) examined the transfer of 12 chromosomes derived from *L. longiflorum* and the other 12 chromosomes derived from an Asiatic hybrid, confirming recombination in parental genomes. In the case of OA hybrids, many of the triploid hybrids show at least some fertility, however, hundreds of progeny plants need to be rescued by embryo rescue obtained from backcrosses with A, OA and mitotically doubled OA hybrids (Barba-Gonzalez et al., 2004). From the hundreds (approximately 700) of the OA hybrids that were produced, only 12 (1.7%) were confirmed to produce 2n pollen (Barba-Gonzalez et al., 2004). Thus far, this method is considered to occur occasionally and very rare in interspecific hybrids of lilies (Barba-Gonzalez, 2012).

Although introgression of desired genes has been successfully achieved through interspecific hybridization, a series of backcrosses is still required to ensure the introgression of the desired trait into appropriate recipients. During subsequent backcross generations, not all the chromosomes will be transferred from the donor plant, and eventually they will be completely lost in further backcrosses. Despite the successful generation, by the techniques mentioned, of hybrids that were not possible to make before, breeders are confronted with several challenges and restrictions in each step, causing the whole breeding program to be slow, time consuming, laborious and without any guarantee of success.

Propagation of commercial lilies

Lilies can be propagated vegetatively by means of bulblets or scales. The small bulblets, either produced at the stem above the ground (bulbils) or around the stem base in the ground (bulbs), can be directly sown for vegetative propagation (Kumar et al., 2008). For commercial trade, lily bulblets are produced from scales. Each excised scale can produce up to 4 bulblets (Askari et al., 2014). However, vegetative propagation can be relatively slow and pathogen infection often results in low-quality bulbs (Krens and van Tuyl, 2011), and thus it is not possible to meet the growing demands for commercial trade with respect to both quantity and quality. Production of a lot of healthy and genetically homogenous plant material requires rapid multiplication by tissue culture techniques. Many explant types including scales, leaves and petioles have been used to regenerate bulblets under *in vitro* conditions (Askari et al., 2014). Among the different *in vitro* systems explored, bulblets regenerated from scales proved to be the best method of choice because scales seem to be most productive (Kumar et al., 2008).

Ornamental traits desirable to improve via genetic modification

The main ornamental trait to be modified in lilies is colour, but in addition, modification of flower scent, and flower and plant morphology are of major interest in

breeding of commercial lilies (Tanaka et al., 2005). These type of traits are visible, so easier to score and breed for (Krens and van Tuyl, 2011). More importantly, visible traits have a great influence on consumer choice when buying ornamental flowering plants (Noda et al., 2017). Other increasingly important traits are yield, postharvest quality and disease resistance, but these type of traits are more difficult to score and breed for (Krens and van Tuyl, 2011).

Flower scent

Although flower scent research is a relatively new field of study, the biochemical pathways leading to flower scent (composed of volatile compounds) are many and complicated. Some of these pathways we are beginning to understand and have been partly elucidated and several genes encoding the enzymes responsible for the synthesis of volatile compounds have been cloned (Dudareva and Pichersky, 2000; Pichersky and Dudareva, 2007). Flower scent is associated with terpenoid, benzenoid and phenylpropanoid compounds (Pichersky and Dudareva, 2007). Terpenoids, especially monoterpenoids such as linalool and (*E*)-*β*-ocimene, which smell fragrant and sweet, are the main scent compounds found in the tepals of Oriental hybrid lilies (Kong et al., 2012), and they can also be found in vegetative tissues as defence compounds (Dudareva and Pichersky, 2000). A *linalool synthase* (*LIS*) gene from *Clarkia breweri* was first isolated by Dudareva (1996). Since then, many more structural genes involved in scent formation have been identified and characterized from other scented flowers such as rose (Guterman et al., 2002), snapdragon (Dudareva, 2003) and petunia (Schuurink et al., 2006). The transfer of flower scent from one species to another species could be possible through genetic modification, but might require the introduction of an entire pathway, hence the transfer of multiple genes. This could limit applications.

Flower or plant morphology

Growth retardation or dwarfism is an important breeding goal for several types of potted plants. Previously, dwarfism could be achieved by the use of chemicals (e.g. chlormequat chloride, mepiquat chloride, chlorphonium, AMO-1618, ancymidol, flurprimidol, tetcyclacis, paclobutrazol, uniconazole-P, and inabenfid) that inhibit the synthesis of gibberellic acid (GA) (Rademacher, 2000). However, some of those chemicals are hazardous to the environment as well as to humans (Campbell et al., 1989; De Castro et al., 2004). Recently, Hirano et al. (2008) reviewed several regulatory genes involved in GA biosynthesis and came to the conclusion that only a few have been actually used for floricultural genetic modification. Among them, the *Arabidopsis GA insensitive* (*gai*) gene that was found to have the ability to greatly supress GA responses causing dwarfism in (pot) chrysanthemum (Petty et al., 2001). Transgenic plants of regal pelargonium exhibit reduced plant height, leaf area, petal area, and corolla length when transformed with the *rol*C gene from *Agrobacterium rhizogenes* (Boase et al., 2004), and finally, a compact *Kalanchoe* has

been produced by Lütken et al. (2010) through the introduction of the *Short internodes* (*SHI*) gene isolated from *Arabidopsis thaliana.*

Identity of floral organs described by the classical ABC model has been recently reviewed by Bowman et al. (2012). The letters represent three overlapping gene classes, named A (*APETALA 2, AP2*), B (*APETALA 3, AP3*) and C (*AGAMOUS, AG*). *AP2* is involved in the formation of sepals in whorl 1, while *AG* regulates the formation of carpels in whorl 4. The overlap of *AP2+AP3* leads to the formation of petals in whorl 2, while *AP3+AG* result in the formation of stamens in whorl 3. The ABC model was first established in dicot model species such as snapdragon (Coen and Meyerowitz, 1991), *Arabidopsis* (Bowman et al., 2012; Gustafson-Brown et al., 1994), petunia (Colombo et al., 1995), and later in some monocot species such as orchid (Hsu and Yang, 2002), tulip (Kanno et al., 2003) and lily (Benedito et al., 2004). Manipulation of floral morphology in lily started much later and is still in its infancy.

Postharvest quality

Lilies are ranked second after tulips among bulbous plants propagated for cut flower production (van der Meulen-Muisers et al., 1999). As cut flowers, the ability to maintain its postharvest quality during distribution and storage is very important to both producer and distributor. Products are often shipped long distances and stored for long periods before they can reach the buyers. The major symptoms that limit the postharvest quality are development of leaf yellowing and browning, abscission of floral buds, lack of flower opening and tepal wilting (van Doorn and Han, 2011), which can be prevented by the use of chemical preservatives. For examples, benzyladenine and gibberellins can prevent leaf yellowing and browning (Han, 2001), ethylene inhibitors like eg. silver thiosulfate (STS) and 1-methylcyclopropene (1-MCP) can improve the quality of cold-stored stems and the opening of buds (Han and Miller, 2003), and inclusion of sugar in vase solutions can improve vase life of Oriental lily 'Stargazer' (Han, 2003). Genetic engineering in physiological processes related to plant and flower quality should aid in producing new cultivars with improved postharvest quality, without the need to use chemicals.

Disease resistance

So far, few studies to determine resistance against *Fusarium, Botrytis* and Lily Mottle Virus (LMoV) in *Lilium* have been reported (Straathof and van Tuyl, 1994; van Heusden et al., 2002; Shahin et al., 2009). *Lilium* species belonging to the section *Sinomartagon* are highly resistant to *Fusarium* and virus, while species belonging to the section *Archelirion* are highly resistant to *Botrytis* (Lim and van Tuyl, 2006). This finding gives rise to a special interest in breeding to produce OA hybrids with a combination of these resistance traits. However, due to a high degree of interspecific incompatibility between sections, the main

obstacle is the mere difficulty in generation of fertile hybrids that are directly useable for further breeding steps. Alternatively, molecular cloning of different disease resistance (*R*) genes from resistant plants enables the transfer of those genes to a non-resistant plant by genetic modification to introduce resistance against the appropriate pathogen (Staskawicz et al., 1995).

Agrobacterium-mediated transformation

Using classical breeding for improvement of specific traits in lilies, is not easy. An alternative is provided by the technology of genetic modification. In this way, existing elite cultivars can be directly equipped with well-characterized genes, maintaining their high quality and adding new, desirable traits. *Agrobacterium tumefaciens* is a natural genetic engineer that is able to transfer a DNA segment from a tumor-inducing (Ti) plasmid to the nuclear genome of host plants (Nester, 2015). Because of this property, *A. tumefaciens* is widely used in genetic modification, amongst others also of flower colour (Katsumoto et al., 2007; Lee et al., 2009; Nakatsuka et al., 2007). *A. tumefaciens* is a gram-negative, soil-borne bacterium that naturally has a broad host range in plants, but mostly dicotyledonous floral plants (Pitzschke, 2013). There still remain, however, many challenges for *Agrobacterium* recalcitrant species, especially monocotyledonous plants as well as woody species (Gelvin, 2003; Hoshi et al., 2004). Lilies are among the monocot species that were thought to be recalcitrant to genetic transformation by *A. tumefaciens* (Wang et al., 2012). Numerous efforts were made to develop an efficient *Agrobacterium*-mediated transformation protocol. So far, both *Agrobacterium* and microprojectile bombardment transformation have been achieved in lilies (Hoshi et al., 2004; 2005; Azadi et al., 2010; Cáceres et al., 2011; Liu et al., 2010). Nevertheless, the transformation efficiencies varied between genotypes and mostly were very low (Wang et al., 2012), although also frequencies up to 20% have been claimed using lipoic acid (LA; Cáceres et al., 2011). Increasing transformation efficiency is therefore important to enable more plant cells to take up the introduced DNA and grow into entire, genetically modified plants.

Genetic modification of flower colour

A foremost objective in lily breeding is to produce cultivars with altered flower colours either by increasing colour intensity or creating a new colour. However, some important colours are still unavailable in certain important ornamental species. For example, vivid red/orange (pelargonidin) flowers do not exist in petunia, delphinium and gentian (Nakatsuka et al., 2007), while lily, rose, carnation and chrysanthemum do not produce blue (delphinidin) flowers (Martens et al., 2003; Brugliera et al., 2013). *L. longiflorum* cultivars

only exist in white flowers (Nakatsuka et al., 2007), so it is believed that creating any colour in *L. longiflorum* would be of potential commercial value. It is nevertheless not easy to create novel flower colours in such ornamental crops by classical breeding due to the deficiency of suitable genetic resources in related species. Like stated for resistances in theory this could also be achieved by genetic modification.

In addition to variation in flower colours, variation in colour patterning and spots (as reviewed by Yamagishi, 2013) are also important features in lily hybrids. Bicolour tepals is a common feature in Asiatic hybrids, and formation of red or dark red spots can be seen in both Asiatic and Oriental hybrids (Yamagishi, 2013). According to Yamagishi et al. (2010), spot pigmentation is caused by the transcriptional regulation of biosynthesis genes; *LhMYB6* is involved in pigmentation at raised spots (Fig. 2, Yamagishi et al., 2010), and a newly discovered allele of *LhMYB12-Lat* appears to be involved in pigmentation at splatter spots (Yamagishi et al., 2014b). The raised spots of lily showed an accumulation of anthocyanin pigments at the tepal regions where the surface rises, and so in increased numbers of parenchymal and epidermal cells (Yamagishi and Akagi, 2013). Meanwhile in the splatter spots, anthocyanin pigments accumulated only in epidermal cells and the surface appears smooth (Yamagishi and Akagi, 2013).

Fig. 2. Raised spots appearance in the whole flower of Oriental hybrid lily cultivar ‘Roberta’ (left), and enlarged image of the raised spots on the adaxial side of inner tepals (right).

Flower colour and anthocyanin

Flower colour is predominantly due to the presence of pigments localized in the petal cells. The three main types of pigments involved in petal colour are the flavonoids

(anthocyanins), carotenoids and betalains (Tanaka et al., 2005). Among those pigments, which are secondary metabolites, flavonoids are the most common ones, they have the widest colour range and are studied extensively (Katsumoto et al., 2007). Besides its function in plant cells, flavonoids are known to represent a source of interest in nutraceutical applications for stimulation of DNA repair and protection against oxidative stress, as well as prevention of cancer (Georgiev et al., 2014; Khan et al., 2010; Pietta, 2000). Flavonoids also demonstrated additional biological activities such as anti-inflammatory action (Kim et al., 2004), antimicrobial (Cushnie and Lamb, 2005), antitumor and anti-HIV activity (Wang et al., 1998). These various biological activities have stimulated many researchers to extract the compounds from various plant parts including flowers for testing (Barreira et al., 2008; Srivastava and Gupta, 2009; Liu et al., 2009).

All flavonoids display a C6-C3-C6 skeleton structure derived from the phenylpropanoid pathway (Chandler and Tanaka, 2007). The various classes of flavonoids mainly depend on the structure of the C-ring, and among them, flavones and flavonols are the colourless compounds while anthocyanins are the coloured pigments (Tanaka et al., 2010). The flavones and flavonols may form a complex with anthocyanins to produce a deeper or bluer colour. For this reason, flavones and flavonols have been described as co-pigments (Goto, 1987). The *Arabidopsis MYB12* transcription factor was found to be a flavonol-specific regulator of flavonoid biosynthesis (Mehrtens et al., 2005). High-performance liquid chromatography analyses showed that *Arabidopsis* mutant *myb12* seedlings contained reduced amount of flavonol, while *MYB12* overexpressing plants displayed an increased flavonol content in the young seedlings as well as leaves (Mehrtens et al., 2005). Flavonol contents were stimulated through an activation of expressions of *chalcone synthase (CHS), chalcone isomerase (CHI), flavonol 3-hydroxylase (F3H)* and *flavonol synthase (FLS),* in which the genes products were for flavonol biosynthesis (Mehrtens et al., 2005). In another study, *Lilium MYB12* which is homologous to *Petunia An2* had been shown to determine anthocyanin pigmentation in Asiatic hybrid lilies flower (Yamagishi et al., 2012). The expression of *Lilium MYB12* was found to be correlated with anthocyanin content, with *Lilium MYB12* directly activated the promoters of *CHS* and *dihydroflavonol 4-reductase (DFR*) in the cultivars 'Montreux' and 'Landini' (Lai et al., 2012).

The general term 'anthocyanin' contains two types of molecules that are anthocyanidin and anthocyanin. Anthocyanidin agylcones are sugar-free, unstable and are the precursors of the true anthocyanin. The three major pigments of anthocyanidin are pelargonidin, cyanidin and delphinidin which are then referred to as pelargonin, cyanin and delphinin respectively after they get converted into the true anthocyanin by the addition of sugar group to stabilize them. Anthocyanins produce red, scarlet, pale yellow, magenta, violet, and blue colours (Qi

et al., 2013). In lily, cyanidin 3-O-β-rutinoside forms a major anthocyanin and cyanidin 3-O-β-rutinoside-7-O-β-glucoside is a minor anthocyanin (Nørbæk and Kondo, 1999). Anthocyanins are synthesized in cytosol, then transported and stored in the vacuole, where the vacuolar pH plays an important role in determining the final visible colouration. Flower colour is generally redder in acidic pH (low) and bluer in higher vacuolar pH (Katsumoto et al., 2007). Although higher (neutral) pH generally gives bluer colour, anthocyanins are less stable at higher pH (Tanaka et al., 2005). Japanese morning glory (*Ipomoea nil*) and petunia have been model species for the study of vacuolar pH. In Japanese morning glory, a structural gene (*Purple)* shown to control vacuolar pH encodes a Na^+/H^+ antiporter (*InNHX1*) (Fukada-Tanaka et al., 2000). The gene was highly expressed and increased the vacuolar pH from 6.5 to 7.5 which gave a more blue appearance. Homologues (*NHX1*) have been isolated from petunia, torenia and *Nierembergia* (Yamaguchi et al., 2001). Also, specific transcription factor *PH4* interacting with *AN1* and *JAF13* play a role in vacuole acidification and flower colour in petunia as was demonstrated by Spelt et al. (2000) and Quattrocchio et al. (2006).

Anthocyanin biosynthetic pathway

The major pathway leading to anthocyanin biosynthesis is generally conserved among higher plant species (Fig. 3). The first enzyme in the pathway, chalcone synthase is encoded by the gene *CHS.* CHS catalyzes the condensation of three malonyl-CoA molecules with one molecule of 4-coumaroyl-CoA as a starter substrate to form naringenin chalcone (Koes et al., 1989). *CHS* generally belongs to a gene family with multiple gene copies (Wannapinpong et al., 2013). The number of gene copies present in this family depends on the plant species. For example, petunia contains at least eight *CHS* gene copies (Koes et al., 1989) and morning glory has six gene copies (Durbin et al., 2000). In Asiatic hybrid lily cultivars 'Montreux' (pink tepals with spots) and 'Connecticut King' (yellow tepals without spots), three *CHS* gene copies (*CHSa, CHSb* and *CHSc*) were identified; *CHSa* and *CHSb* are expressed in tepals, whereas *CHSc* is specific to anthers (Nakatsuka et al., 2003). The expression of *CHSa, CHSb* and *CHSc* were not detected in vegetative organs such as leaves, stems and white bulbscales in both cultivars. However, the three genes were expressed in the red bulbscales, in which anthocyanin formation was stimulated by light (Nakatsuka et al., 2003). Despite the considerable number of *CHS* genes in plants, mutations in a single *CHS* gene are responsible for white colouration in morning glory (Durbin et al., 2000). Chalcone isomerase (CHI) subsequently catalyzes the isomerization of naringenin chalcone (tetrahydroxychalcone) to naringenin (Brugliera et al., 2013). Two *CHI* genes (*CHIa* and *CHIb*) are known to be involved in anthocyanin biosynthesis in the flowers of petunia (van Tunen et al. 1988; 1989) and lily (Suzuki et al., 2015). Flavanone 3-hydroxylase (F3H)

converts naringenin into dihydrokaempferol (Brugliera et al., 2013). In *Arabidopsis, F3H* appeared to be a single copy gene, and it's expression is co-ordinately regulated with *CHS* and *CHI* (Pelletier and Winkel-Shirley, 1996).

Flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H) are two important enzymes responsible to catalyze dihydrokaempferol (i.e. precursor of brick-red/orange pelargonidin) conversion to dihydroquercetin (i.e. precursor of red/pink cyanidin) and dihydromyricetin (i.e. precursor of blue/violet delphinidin), respectively (Grotewold, 2006). Thus, the establishment of *F3'H* and *F3'5'H* genes encoding the enzymes is essential for diverse flower colours. Dihydroflavonol 4-reductase (DFR) converts each of the three dihydroflavonols – dihydrokaempferol (DHK), dihydroquercetin (DHQ) and dihydromyricetin (DHM) – to leucoanthocyanidins (Wang et al., 2013). The structural similarity between the three dihydroflavonols, differing only in the number of hydroxyl groups on the B phenyl ring, which is not a site of enzymatic action is presumably a reason why DFR in general can utilize all the three dihydroflavonols as substrates (Helariutta et al., 1993; Meyer et al., 1987; Tanaka et al., 1995). However in some genera, like *Gerbera*, *DFR* is able to reduce all the three substrates (Johnson et al., 2001), while in other genera, like *Petunia* (Forkmann and Ruhnau, 1987), *Cymbidium* (Johnson et al., 1999) and *Freesia* (Li et al., 2017), *DFR* could not accept DHK as substrate, hence they could not produce brick-red/orange pelargonidin-type anthocyanin in their flowers. Apparently, Bashandy and Teeri (2017) discovered orange petunias in flower benches in the city of Helsinki, Finland, and it was found that they expressed the maize *A1* gene encoding DFR (Meyer et al., 1987) and thus were genetically modified. 30 years have passed since the gene was genetically introduced into petunia. Over the years, many *DFR* genes from various plants have been characterized (Chu et al., 2014; Helariutta et al., 1993; Johnson et al., 1999). *Lilium speciosum* with mutated *DFR* display white flower colour (Suzuki et al., 2015). As the final step in the biosynthesis, anthocyanidin synthase (ANS) catalyzes the final oxidation of the colourless leucoanthocyanidins to coloured anthocyanidins – pelargonidin, cyanidin and delphinidin (Nakajima et al., 2001).

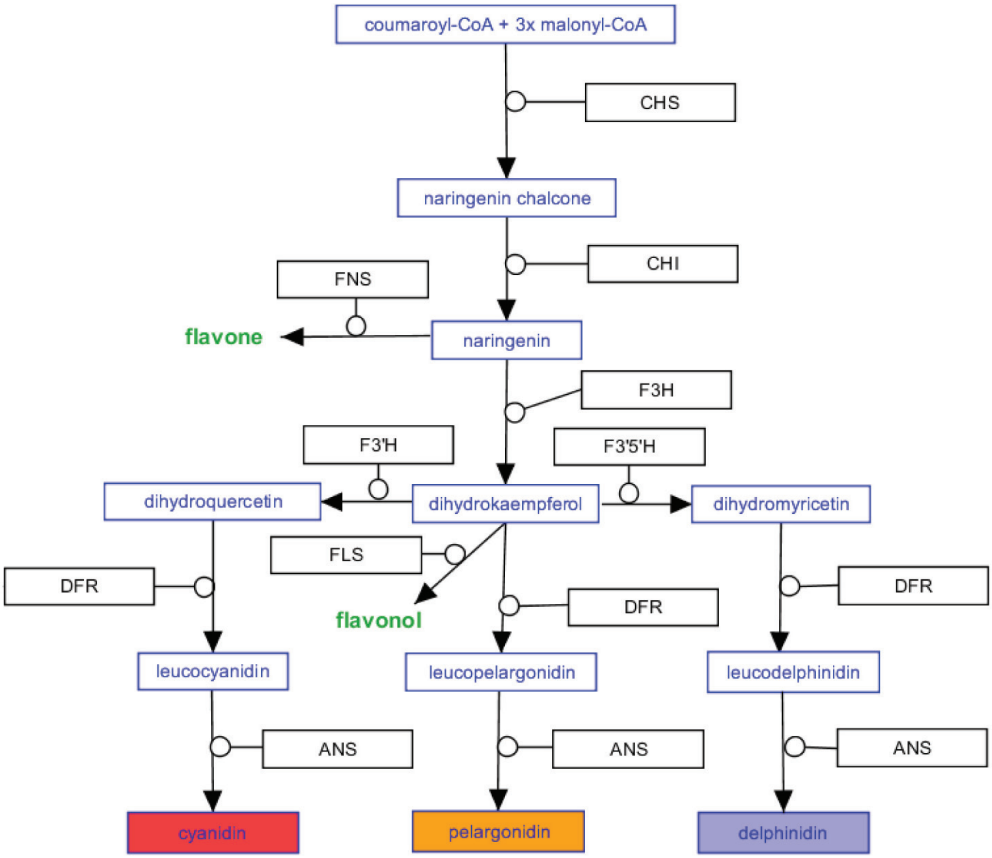

Fig. 3. Schematic diagram of anthocyanins biosynthetic pathway relevant to flower colour (modified based on (Katsumoto et al., 2007; Tanaka et al., 2009)). CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; FLS, flavonol synthase; FNS, flavone synthase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase.

Genetic modification of flower colour

Successful genetic modification of plants required three technical elements including isolation of functional genes, the development of an efficient transformation system for a target species, and regulation of gene expression in the target species (Tanaka et al., 2010). Numerous articles describing isolation of many genes related to flower colour, establishment of transformation systems in various plants, and genetic modification of flower colour have been previously reviewed (Chandler and Tanaka, 2007; Tanaka et al., 2005; Tanaka, 2006; Tanaka and Ohmiya, 2008; Tanaka et al., 2010; Yamagishi, 2013).

In this study, we are focussing on manipulation of transcription factors and structural genes for modification of flower colour in *Lilium* spp.. The activitiy of anthocyanin biosynthetic enzymes is mainly regulated at the transcriptional level, thus manipulation of transcription factors is an ideal strategy to alter the expression of multiple target genes. According to Koes et al. (2005), *R2R3-MYB* usually interacts with *basic helix-loop-helix (bHLH)* and *WD40* transcription factors to regulate the transcription of anthocyanin biosynthetic genes in flower organs. Among these three transcription factors, *MYB* has been most extensively studied and was shown to play the most important role in regulating anthocyanin production, as it can bind directly to the target genes (Hichri et al., 2011). In *Antirrhinum majus* flowers, two transcription factors, a *MYB*-type *ROSEA1* (*ROS1*) and a *bHLH*-type *DELILA* (*DEL*) interact to induce anthocyanin biosynthesis (Schwinn et al., 2006; Goodrich et al., 1992). Maligeppagol et al. (2013) also reported that transgenic tomato plants accumulated higher amounts (70–100 fold) of anthocyanin in the fruit when the transcription factors, *ROS1* and *DEL* isolated from *A. majus* were expressed. In our experiments, *N. benthamiana* is a white-flowered model plant species which is easily transformable. Here, the mutations or deficiencies leading to the white colour are unknown, as they are in the *L. longiflorum* cv. 'Lincoln'. On the other hand, the flowers of Oriental hybrid lily cv. 'Rialto' are thought to be white due to a single amino acid (W-to-L) mutation in the R2-repeat region of *MYB12* transcription factor (Yamagishi, 2011; Yamagishi et al., 2012). A similar mutation has been found in *MYB12* of *L. speciosum* having white tepals and red anthers (Suzuki et al., 2015). The non-functioning status of the *L. speciosum MYB12* gene was experimentally confirmed in *N. benthamiana* transient expression assays (Suzuki et al., 2015). Since *MYB12* is functionally interchangeable with *ROS1*, we hypothesize that it would be possible to produce red flowers by introducing the *ROS1* and *DEL* transcription factors in 'Rialto'. The effects of these regulatory genes individually and in combination were investigated in *N. benthamiana* and *Lilium* spp. flowers. This is described in **Chapter 3** of this thesis.

For successful flower colour engineering, tissue-specific promoters that activate the expression of a target gene specifically at the desired tissues are required. *Floral Binding Protein 1* (*FBP1*) is a flower-specific promoter which is expressed in the petals and stamens of *Petunia hybrida* (Angenent et al., 1993). The *FBP1* promoter has been used to study flower senescence in carnation and herbicide resistance in lily (Bovy et al., 1999; Benedito et al., 2005). The observation that *FBP1* was active in petals and stamens from the primordia stage to mature flowers of carnation indicated that this promoter is still active in the later stages of flower development when anthocyanin is produced. Because of this property, *FBP1* is a suitable candidate to serve as flower specific promoter in genetic modification of dicot species, and possibly of monocot species.

Although white is also an economically important flower colour, only a few studies investigating the genetic background responsible for white colouration in lilies have been

reported. Molecular mechanisms regulating white colouration in lilies varied depending on the plant genotype being considered. For example, the white colouration in Asiatic hybrid lily cultivars 'Navona' and 'Silver Stone' was due to insufficient expression of the *MYB12* transcription factor gene (Yamagishi et al., 2012). Moreover, *MYB12* was not expressed at all in tepals of Oriental hybrid lily cultivar 'Casa Blanca' and its putative parent *Lilium auratum* var. *platyphyllum,* causing their tepals to be white (Yamagishi et al., 2014a). In the Oriental hybrid lily cultivar 'Rialto', *MYB12* was expressed, but a W-to-L amino acid substitution was detected in the R2-repeat, which likely disrupted its function (Yamagishi, 2011; Yamagishi et al., 2012). However, the wild species *Lilium speciosum* white/red line (i.e. white tepals and red anthers) contained a *MYB12* gene that was identical to that of 'Rialto'. First, this occurrence was considered to be the cause of white tepals in the *L. speciosum* white/red line. However, in the white/yellow line (i.e. white tepals and yellow anthers), a nonsense mutation in *DFR* was identified which led to a premature stop codon and supposedly caused the white tepal and yellow anther phenotype (Suzuki et al., 2015). This indicated that there are several mechanisms including mutations, absence of genes, and differential expression levels of various genes that generate white flowers in lilies. Determination of the genetic background of the host plants is, therefore, one of the most fundamental steps needed to be completed before a sound genetic modification strategy can be set up.

Outline of the thesis

The aims of the research described in this thesis were to study anthocyanin as flower colour determinant in *Lilium* spp., and to alter lily flower colours via genetic modification. However, *Lilium* is recalcitrant to *Agrobacterium*-mediated transformation, and thus exhibits a low transformation efficiency. *Lilium* transformation takes at least 8 to 10 months starting from untransformed callus until the first transgenic *in vitro* plantlets can be obtained, with no guarantee of success. To reach the stage in which flowering will occur, another 18 months are necessary for bulb growth (year 1), followed by a cold treatment and subsequent growth resulting in a flower (year 2). Therefore, a simple gene transfer system allowing rapid evaluation of the genes under investigation is required, preferably without the need of going through the entire transformation and regeneration and flower production processes.

To enhance *Agrobacterium*-mediated transformation efficiency, we investigated the effects of increasing concentrations of paclobutrazol (PBZ) and 2-amidoindane-2-phosphonic acid (AIP) as salicylic acid (SA) inhibitors and lipoic acid (LA) as antioxidant and stress alleviator in culture media during transformation (**Chapter 2**). Two model plants, *P. hybrida* and *N. benthamiana* were used in this experiment because they are relatively easy to transform. Adding SA always decreased the transformation efficiencies in both plant species, confirming the role of SA in plant defence responses against *Agrobacterium*. The effect of the inhibitors on the transformation efficiency was significant but not consistent for

concentration or type for the two plant species studied. PBZ at 10 µM resulted in the highest transient and stable transformation efficiencies in *P. hybrida*. However in *N. benthamiana*, 50 µM AIP showed a significant increase in transient transformation, but the highest stable transformation was achieved at 10 µM AIP. LA at 10 µM significantly increased transient expression in *P. hybrida*, however, it was without any effect in *N. benthamiana*. Although the effectivity of the SA inhibitors could not be confirmed by measuring free SA levels, indications have been found that the SA inhibitors and LA potentially can enhance transformation efficiency in dicot species, and possibly in monocot species too.

Chapter 3 describes the effect of introducing *ROSEA1* (*ROS1*, a MYB-type) and *DELILA* (*DEL*, a bHLH-type) transcription factors from snapdragon under control of a flower specific promoter, *Floral Binding Protein 1* (*FBP1*) from petunia into *N. benthamiana* and *Lilium* spp. The usefulness of the *FBP1* promoter was demonstrated by the generation of normal growing *N. benthamiana* plants carrying purplish flowers, while the use of the CaMV *35S* promoter led to the development of stunted plants with anthocyanins in all plant parts. But first, a simple transient transformation using agroinfiltration was developed for lily tepals. PBZ at 10 µM enhanced *GUS* expression in lily tepals, confirming the positive effect of SA inhibitors on transformation efficiency (**Chapter 2**). The *ROS1* and *DEL* transcription factors, expressed under control of the *FBP1* promoter are functional in flowers and able to regulate biosynthetic pathways leading to delphinidin and cyanidin accumulation in flowers of *N. benthamiana* and the Oriental hybrid lily cv. 'Perth', respectively. No pigmentation was induced in the white flowered *L. longiflorum* cv. 'Lincoln' and Oriental hybrid lily cv. 'Rialto', suggesting the absence of or mutation in the structural genes.

Chapter 4 describes the genetic background behind white colouration in lily cultivars 'Lincoln' and 'Rialto', and molecular mechanisms regulating flower colour, especially those involved in the anthocyanin biosynthesis pathway. The white colour of 'Lincoln' flowers is likely caused by the absence of two structural genes (*F3'H* and *DFR*) and one transcription factor gene (*bHLH2*). In 'Rialto', *DFR* was present but not expressed. Together with that, *ANS* is hardly expressed in both cultivars. High amounts of dihydrokaempferol accumulated in tepals of all four lily cultivars indicating the expression and functionality of the early structural genes up to *F3H* in the pathway. The elevated expression of the structural genes is strongly correlated with the expression of *MYB12* and *MYB15*. In contrast, *bHLH2* was present but hardly expressed in Oriental hybrid lily cv. 'Gran Tourismo', a cultivar with pronounced red tepals.

In **Chapter 5**, *F3'H, DFR, ANS* and *MYB12* genes which were lacking or hardly expressed in 'Lincoln' and 'Rialto' were isolated from the red flowers of 'Gran Tourismo', and several combinations of the genes and transcription factors (including *ROS1* and *DEL*, from **Chapter 3**) were introduced and expressed in the white flowers of 'Lincoln' and 'Rialto' to complement the genes. Almost all individual isolated lily genes seemed functional in the

pink flowered Oriental hybrid lily cv. 'Perth' and in *Arabidopsis* mutants, except for *DFR*. Co-introduction of multiple genes failed to induce anthocyanin biosynthesis in 'Lincoln' and 'Rialto' most probably due to the complexity of anthocyanin biosynthetic pathway, in which *DFR* seemed to be the main bottleneck.

Chapter 6 summarizes all findings from the above experimental chapters and discusses the potential of these findings as a substantiated foundation for the molecular breeding of new, originally white lily cultivars with novel flower colour.

CHAPTER 2

Enhancing *Agrobacterium*-mediated transformation efficiency using salicylic acid inhibitors and lipoic acid in *Petunia hybrida* and *Nicotiana benthamiana*

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Abstract

Salicylic acid (SA), an important phytohormone involved in plant defence mechanisms against plant pathogens might play a role in determining *Agrobacterium*-mediated transformation efficiency. A conflict between *Agrobacterium* and the host plant likely causes a low transformation efficiency. To enhance *Agrobacterium*-mediated transformation efficiency, we investigated the effect of adding SA, paclobutrazol (PBZ) and 2-amidoindane-2-phosphonic acid (AIP) as SA inhibitors and lipoic acid (LA) as antioxidant and stress alleviator in culture media during the transformation of *Petunia hybrida* Vilm. and *Nicotiana benthamiana* Domin. Adding SA always decreased the transformation efficiencies in both plant species; this could confirm the role of SA in the plant defence mechanisms against *Agrobacterium*. The effect of the inhibitors on transformation efficiency was significant but not consistent for concentration or type for the two plant species studied. In *P. hybrida*, 10 µM PBZ resulted in the highest transient and stable transformation efficiencies. However in *N. benthamiana*, 50 µM AIP showed a significant increase in transient transformation, but the highest stable transformation was achieved at 10 µM AIP. LA at 10 µM significantly increased transient expression in *P. hybrida*, however it was not efficient in *N. benthamiana*. Indications have been found that the SA inhibitors and LA potentially can enhance transformation efficiency.

Keywords: *Agrobacterium*-mediated transformation, salicylic acid, inhibitors, lipoic acid, *Petunia hybrida, Nicotiana benthamiana*

Introduction

Genetic modification of most plants is done via *Agrobacterium*-mediated transformation, which remains the most preferred method over the direct DNA delivery systems because of its ability to transfer large segments of DNA with stable integration and consistent expression (Barampuram and Zhang, 2011; Untergasser et al., 2012). Two partners involved in *Agrobacterium*-mediated transformation, the plant and the *Agrobacterium*, however have conflicting interests. The plant wants to fight off the *Agrobacterium,* and the *Agrobacterium* wants to colonize the plant. This conflict likely causes a low transformation efficiency in many plant species, either by efficient defence of the plant or by low survival of the plant after the stressful exposure. Therefore, a successful gene transfer event requires a balanced interaction between both partners, the plant and *Agrobacterium*.

Salicylic acid (SA) is a major phytohormone involved in induced defence responses against biotrophic pathogens including *Agrobacterium* (Chen et al., 2009; An and Mou, 2011; Zhu et al., 2014). Plants potentially synthesize SA through two pathways, via either phenylalanine or isochorismate (Fig. 1). Phenylalanine ammonia lyase (PAL) and isochorismate synthase (ICS) are key-enzymes involved in the synthesis of SA in the pathways, respectively (Wildermuth et al., 2001; Chen et al., 2009; Vlot et al., 2009). In *Arabidopsis*, mutations in the *ICS1* gene results in almost a complete loss of pathogen-induced SA accumulation (Wildermuth et al., 2001), while analysis of the quadruple PAL mutant showed 50% reduction in total SA after pathogen infection (Huang et al., 2010). It seems that the isochorismate pathway contributes to a major SA accumulation during plant defence mechanisms (Wildermuth et al., 2001). However, evidence for a potential involvement of the phenylalanine pathway in plant defence has been extensively reported (León et al., 1995; Meuwly et al., 1995; Mauch-Mani and Slusarenko, 1996; Dong et al., 2014). The expression of PAL is rapidly induced during pathogen infection, and inhibition of the PAL activity results in *Arabidopsis* plants which are completely susceptible to the causal pathogen of downy mildew, *Peronospora parasitica* (Mauch-Mani and Slusarenko, 1996). Isotope feeding experiments indicate that the PAL enzymatic pathway contributes to mainly pathogen-induced SA accumulation in tobacco (León et al., 1995; Meuwly et al., 1995). Similarly, under chilling stress conditions, gene expression and enzymatic activities of PAL and benzoic acid 2-hydroxylase (BA2H) are highly induced in comparison to the ICS (Dong et al., 2014). Based on these combined evidences, it was commonly accepted that the PAL pathway plays an important role in plant defence mechanisms against biotic and abiotic stresses.

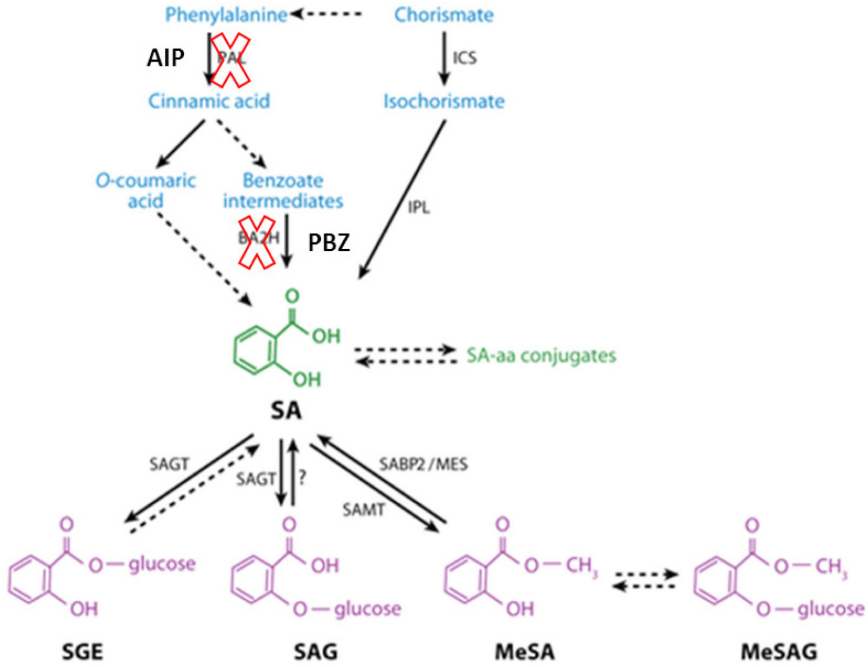

Fig. 1. Pathways for the biosynthetic origin of salicylic acid involved in plant defence mechanisms as derived from Vlot et al. (2009) and where blocking is supposed to take place. Abbreviations for the enzymes: phenylalanine ammonia lyase (PAL), isochorismate synthase (ICS), isochorismate pyruvate lyase (IPL), benzoic acid 2-hydroxylase (BA2H), salicylic acid (SA), SA glucosyltransferase (SAGT), amino acid (aa), SA methyltransferase (SAMT), SA-binding protein 2 (SABP2), methyl esterase (MES), salicyloyl glucose ester (SGE), SA *O*-*β*-glucoside (SAG), methyl salicylate (MeSA). methyl salicylate *O*-*β*-glucoside (MeSAG); and the blockers: paclobutrazol (PBZ), 2-amidoindane-2-phosphonic acid (AIP). Established interactions are indicated by solid lines, hypothesized or less well characterized interactions are shown in dashed lines.

Inhibition of enzymes involved in PAL pathways may reduce SA accumulation and consequently increase the plant's susceptibility to pathogens. In this way, exposure to *Agrobacterium tumefaciens* could result in more plant cells being transformed. The PAL enzyme catalyzes the first enzymatic step in the phenylalanine pathway. The 2-aminoindan-2-phosphonic acid (AIP), a specific competitive PAL inhibitor (Zoń and Amrhein, 1992) reduces SA accumulation which affects resistance in multiple plant species such as cucumber, potato and *Arabidopsis* (Coquoz et al., 1998; Mauch-Mani and Slusarenko, 1996; Meuwly et al., 1995). In addition, inhibition of PAL through the use of AIP has been reported to reduce

phenylpropanoid biosynthesis, resulting in reduced oxidative browning in plant tissue culture (Jones and Saxena, 2013). This compound, however, hinders the plant growth of duckweed, *Spirodela punctata* by disturbing phenylpropanoid biosynthesis and stimulation of excessive starch accumulation (Janas et al., 1998). Nevertheless, it was argued that the use of AIP displayed no marked effects on the cell growth of a traditional Chinese medicinal herb, *Cistanche deserticola* (Hu et al., 2011), and indeed increased tissue growth by controlling oxidative browning in *Artemisia annua* (Jones and Saxena, 2013).

Paclobutrazol [2RS, 3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1.2.4-triazol-1-yl)pentan-3-ol] (PBZ) is a plant growth regulator that can suppress gibberellin biosynthesis and cause several morphological and physiological changes in plants including increased photosynthetic pigments, improved nutrient uptake, retarded senescence, enhanced flowering, seed and oil yields (Kumar et al., 2012). In the final step of the phenylalanine pathway, benzoic acid 2-hydroxylase (BA2H) converts benzoic acid to SA, and PBZ is known as the inhibitor of BA2H (León et al., 1995). The activity of BA2H has been detected in multiple plant species including tobacco (León et al., 1995), rice (Sawada et al., 2006), wheat (Xiang et al., 2011) and cucumber (Dong et al., 2014). In tobacco, for example, the activity of BA2H is strongly induced in response to tobacco mosaic virus (TMV) infection. Inhibition of BA2H through the use of 20 µM PBZ resulted in complete loss of BA2H activity in tobacco, however, SA content was not determined (León et al., 1995). In wheat seedlings, SA accumulation is slightly reduced when a higher concentration (100 µM) of PBZ is applied (Xiang et al., 2011). Because PBZ could inhibit the activity of BA2H leading to reduced SA biosynthesis, we hypothesize that PBZ could also play a role in inhibiting SA production upon plant-pathogen infection.

In plants, browning and necrosis of tissues after inoculation with *Agrobacterium* is likely to occur. The browned tissues suffer oxidative stress which occurs when reactive oxygen species (ROS) are not rapidly scavenged and the rate of repair of damaged cell components fails to keep pace with the rate of damage (Mullineaux and Baker, 2010). As a result, the browned tissues usually are slower to recover after co-cultivation and eventually cell death can occur. The effects of antioxidants and anti-stress compounds including glycine betaine, glutathione, polyvinylpyrrolidone and LA have been previously reported (Dutt et al., 2011; Uchendu et al., 2010). Glycine shows moderate beneficial effect while glutathione and polyvinylpyrrolidone did not show any beneficial effect on *Agrabacterium*-mediated transformation efficiency. Lipoic acid (LA) has antioxidant properties and the ability to scavenge ROS in animals; it has been reported as a general transformation enhancer in soybean, wheat, tomato and cotton by reducing tissue browning, increasing transient gene expression and reducing escapes (Dan et al., 2009). Dan and his coworkers (2009) showed that LA at 5 µM did significantly reduce the number of explants having a high level of tissue browning (2-fold) and 10 µM significantly increased (3-fold) transient transgene

expression and subsequently increased the number of transgenic plants produced in tomato transformation. In another report, the use of 50 mM LA increases efficiency (20%) in *Lilium* transformation (Cáceres et al., 2011). Thus it is imperative to re-evaluate the use of LA in *P. hybrida* and *N. benthamiana* as model systems.

Our preliminary work suggested the importance of SA in *Agrobacterium*-mediated transformation efficiency (Hasan Nudin et al., 2015), but it was still unclear how SA inhibitors participate in changes in SA level in the plant tissues. In this paper, we investigated the effects of SA, SA inhibitors and LA on transient and stable *Agrobacterium*-mediated transformation efficiencies in *P. hybrida* and *N. benthamiana*. Adding SA in culture media during transformation would increase the plant defence hence lower the transformation efficiency. In contrast, adding SA inhibitors could reduce plant defence hence increase the transformation efficiency. Adding LA could allow the plant cells to cope better with stressful pathogen attack and become more receptive for transformation. Optimal concentrations of the inhibitors and LA for transformation were determined. In addition, we investigated changes in the free SA level of plant tissues as a consequence of adding SA or its inhibitors.

Materials and Methods

Plant materials

P. hybrida (*Ph*) line W115 and *N. benthamiana* (*Nb*) were chosen because they are model species in *Agrobacterium*-mediated transformation, providing a benchmark level for efficiency; they allow rapid evaluation of the effects of inserted reporter and selectable marker genes in transgenic plants. *P. hybrida* was sown in pots (10.5 cm diameter) in the greenhouse. The greenhouse temperature was maintained at around 21˚C during the day and 19˚C during the night (16/8 h photoperiod). The relative humidity was set at 70%. The soil mixture used in this study was composed of 5% of Swedish sphagnum peat, 41% of grinding clay granules, 5% of garden peat, 4% of beam structure, 33% of steamed compost and 12% of PG-Mix 15-10-20. *N. benthamiana* was grown and propagated *in vitro* on MS30 medium (4.4 g/L Murashige and Skoog medium (MS) salts and vitamins (Duchefa, Haarlem, The Netherlands), 30 g/L sucrose, pH adjusted to 5.8) in white light (Philips, TL-D 36W/840) at 24-26˚C. Fresh and healthy leaves were harvested after four to six weeks after sowing (*Ph*) or four to five weeks after transfer to fresh medium (*Nb*) for transformation.

Agrobacterium-mediated transformation

A. tumefaciens strain AGL1 pBinGlyRed-Asc1 (Zhu et al., 2016) was used for transformation containing the *dsRed* gene (Baird et al., 2000) under the control of the cassava vein mosaic virus (CVMV) promoter as reporter for gene transfer and the *nptII* gene for kanamycin resistance as a selectable marker for monitoring stable transformation. In addition,

two jojoba genes, *ScFAR* and *ScWS* under the control of the glycinin promoter were present, however they are not relevant for this study (Fig. 2). The *Agrobacterium* strain was used both in *P. hybrida* and *N. benthamiana* transformation. The bacteria culture in liquid Luria-Bertani broth (LB) medium with 50 mg/L kanamycin and 50 mg/L rifampicin was grown overnight at 28°C in Innova 4000 incubator shaker (New Brunswick Scientific, UK) at 150 rpm to an OD_{600} ± 1.0. The bacteria were then collected by centrifugation in a Heraeus Multifuge 3S (Kendo Laboratory Products, Germany) at 3000 rpm for 15 mins.

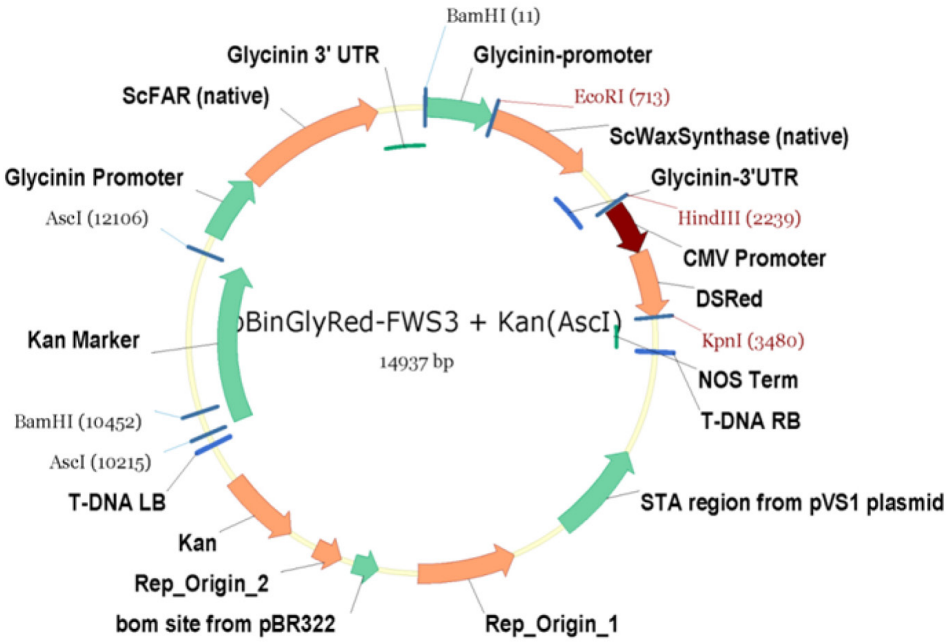

Fig. 2. Binary vector pBinGlyRed-Asc1 containing a *dsRed* reporter gene, two jojoba genes (*ScFAR* and *ScWS*), and the neomycin phosphotransferase II (*nptII*) as a selective marker denoted here as Kan Marker. CMV=cassava vein mosaic virus (CVMV) promoter.

For *P. hybrida,* the transformation method was derived from Horsch and Klee (1986) with minor modifications. The protocols used have been proven to lead to verified stably transformed plants. The leaves were sterilized with 1% (w/v) sodium hypochlorite (NaClO) and 0.1% (v/v) Tween 20 for 10 minutes followed by rinsing three times with sterile distilled water. Leaf discs of about 0.5-1 cm^2 were cut excluding the leaf margin and midrib, and placed upside down on regeneration medium (4.4 g/L MS, 30 g/L sucrose, 4.44 µM benzylaminopurine (BAP), 0.54 µM naphthalene acetic acid (NAA), 0.8% (w/v) micro agar, pH adjusted to 5.8), with addition of 100 µM (final concentration) acetosyringone after autoclaving for 2 days pre-treatment. Leaf discs were inoculated with *Agrobacterium*

suspended in liquid MS30 (4.4 g/L MS, 30 g/L sucrose, pH adjusted to 5.8, with addition of 100 µM acetosyringone (AS) after autoclaving) with OD_{600} ± 1.0 for 1 minute. The discs were blotted dry on sterile filter paper and placed back on the same medium used for pre-treatment for further co-cultivation in the dark at 24-26˚C. After that, the discs were transferred to selection medium, prepared the same as above but lacking AS and containing 200 mg/L cefotaxime, 100 mg/L timentin and 200 mg/L kanamycin.

For *N. benthamiana*, leaf discs of about 0.5-1 cm^2 were cut excluding the leaf margin and midrib. The discs were placed upside down and floating in a Petri dish containing 5 mL liquid callus inducing medium (CIM) (4.4 g/L MS, 30 g/L sucrose, 0.89 µM BAP, 5.37 µM NAA, pH adjusted to 5.8), with addition of 100 µM AS after autoclaving with *Agrobacterium* (OD_{600} ± 0.1). The petri dishes were then incubated in the dark at 24-26˚C for 3 days co-cultivation. After that, the discs were blotted dry with sterile filter paper before placing them upside up to petri dishes containing shoot inducing medium (SIM) (4.4 g/L MS, 30 g/L sucrose, 4.44 µM BAP, 0.54 µM NAA, 8 g/L agar, pH adjusted to 5.8, with addition of 500 mg/L cefotaxime and 50 mg/L kanamycin after autoclaving) for selection.

For both plant species, separate experiments were carried out to test the effects of each compound (AIP, PBZ, SA and LA) in increasing concentrations (0, 1, 10, 50, 100 µM). A stock solution of 10 mM AIP (Ark Pharm, Inc., USA), 50 mM each of PBZ (Duchefa Biochemie B.V., The Netherlands), SA (Duchefa Biochemie B.V., The Netherlands) and LA (Acros Organics, The Netherlands) were prepared in 80% ethanol. The stock solutions were kept in -20˚C until use. A suitable volume of stock solution was added to 500 mL of the media after autoclaving to obtain desired final concentration. The compounds were added in pre-treatment (only *Ph*), co-cultivation and selection media during the first 14 days only. Three petri dish replicates each containing 15 leaf discs which made a total of 45 discs were used for each compound and concentration. After that, all the discs were transferred to fresh selection media without any compound and acetosyringone every 2 weeks for further evaluation. The regenerated shoots were then transferred to rooting media consisting of 4.4 g/L MS, 30 g/L sucrose, 8 g/L micro agar, pH adjusted to 5.8, with addition of 250 mg/L cefotaxime and 200 mg/L kanamycin after autoclaving for *P. hybrida,* and 4.4 g/L MS, 30 g/L sucrose, 0.29 µM indole acetic acid (IAA), 50 mg/L kanamycin for *N. benthamiana*. pH of all media was adjusted to 5.8. Only one shoot per explant was transferred to rooting media. It is therefore highly likely that each regenerated plant represents an independent transformation event.

Statistical data analysis

The transient transformation efficiency (TTE) was determined by counting the number of red fluorescing spots on each treated explant under a stereo microscope with UV light at 14 days after co-cultivation and calculating the mean (Fig. 3 A-B). The higher the number of spots, the higher the gene transfer efficiency, which means also a higher transient

transformation efficiency. We determined the stable transformation efficiency (STE) from the number of explants with fluorescent calli surviving on kanamycin selection media at 30 days of culture (Fig. 3 C-D). Data on TTE and STE were subjected to one-way analysis of variance (ANOVA), and means were compared using Duncan's multiple range test at $P<0.05$. The statistical analysis was performed by SPSS Statistics version 22.

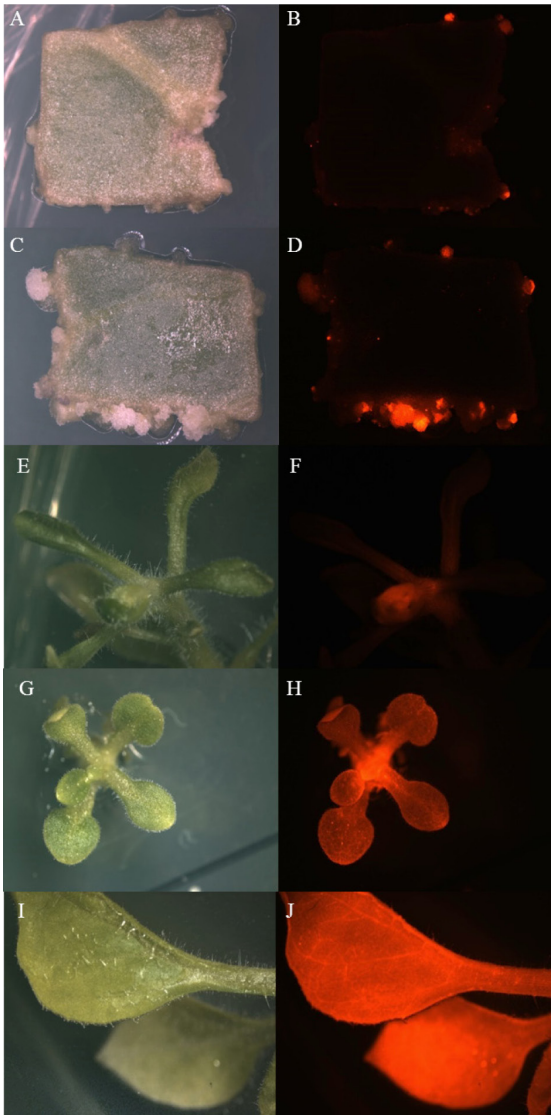

Fig. 3. White light and fluorescent images of *P. hybrida* (A-B) transformed explant with transiently red fluorescing spots; (C-D) stably transformed explant with fluorescent calli on kanamycin-containing medium; red-positive shoot showing (E-F) barely red, (G-H) red, (I-J) very red fluorescing colour.

PCR analysis

Total genomic DNA was isolated from leaves of four or five representative plant samples of putatively transformed fluorescent shoots, non-fluorescent shoots and non-transformed controls according to the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). The PCR amplification mixture contained 100 ng of each sample's genomic DNA as a template, 2.5 mM 10X PCR buffer, 400 µM dNTP, 0.05 U/µL Dreamtaq polymerase (Thermo Fisher Scientific), 10 µM primer *npt*_Fw (5'-GAAGGGACTGGCTGCTATTG-3'), 10 µM primer *npt*_Rev (5'-AATATCACGGGTAGCCAACG-3') that amplify a 423bp fragment of the kanamycin (*nptII*) gene. The PCR amplification was performed using a ABI (Applied Biosystems) thermal cycler under the following conditions: 1 min at 95˚C, followed by 30 cycles of 30 sec at 95˚C, 30 sec at 56°C and 45 sec at 72˚C, with final extension for 7 min at 72˚C. Amplified products were visualized by electrophoresis on a 1% (w/v) agarose gel.

Extraction and purification of free SA

To determine whether or not SA was effectively absorbed from the medium by the explants and whether PBZ did inhibit the accumulation of SA, four replicates of SA- or PBZ-treated leaf discs of petunia were analyzed for free SA content (free SA refers to the part of the total SA synthesized that is not conjugates). For each replicate, five to six inoculated leaf discs were combined and ground in liquid nitrogen after the 14 days treatment. Samples were prepared according to Meuwly and Métraux (1993). About 25 mg of ground sample was extracted in 2 ml of the first solvent (CH_3OH:H_2O:formic acid = 80:20:0.1, v/v/v), containing deuterium labelled free standard of SA (100 pmol/mL). Samples were sonicated for 10 min and shaken for 2 hours at 4°C in darkness. After centrifugation (10 min, 2000 rpm, 4°C, swinging bucket rotor-type) the supernatant was collected and the pellet was re-extracted in 2 ml of the second solvent (acetone:formic acid, 100:0.1, v/v) for 2 hours at 4°C in darkness. Pooled supernatants were evaporated to dryness under vacuum. The extracts were suspended in 1 mL of aqueous methanol (CH_3OH:H_2O=25:75, v/v) and sonicated for 10 min. Plant extracts were purified on the Oasis-WAX column (30mg). The column was activated with 1 mL of CH_3OH, followed by 1 mL of water. After loading 1 ml of the sample, the column was washed with 1 mL of water. SA was eluted with two sequential eluents, 0.7 mL of CH_3OH and 3 mlL of 5% NH_4OH in CH_3OH (pH 12.2). Both fractions were combined and dried under vacuum.

Quantitative analysis of free SA

Quantitative analysis of free SA was done using an Acquity UPLC® System (Waters) coupled with Xevo™ TQ (Waters), triple quadrupole mass spectrometer following Saika et al., (2007). Purified samples were suspended in 200 µl of aqueous methanol

(CH_3OH:H_2O=25:75, v/v), filtered through 0.45 µm PTFE membrane filter (Phenomenex), and injected onto an Acquity UPLC HSS column (50 x 2.1 mm, 1.8 µm, Waters). For each sample three biological replicates were prepared and analysed separately. Salicylic acid was eluted by a binary gradient, consisting of 0.1% formic acid in water (A) and 0.1% formic acid in CH_3OH (B), for 8 min at constant flow rate, 0.5 mL/min at 40°C of analytical column temperature. The gradient elution was performed as follows: 0~4.0 min, 12 % eluent B; 4.0~5.0 min, 12 to 60 % eluent B; 5.0~6.0 min, 60 to 90 % eluent B; 6.0~7.0 min, 90 to 98% eluent B; 7.0~8.0 min, 12 % eluent B. At the end of gradient, the column was equilibrated to initial conditions for 1.0 min. The effluent was introduced in electrospray ion source of mass spectrometer with operating parameters: ESI-negative mode, capillary voltage 3 kV, cone voltage 22 V, source/desolvation temperature 150°C/ 650°C, cone/desolvation gas flow 50/1000 (L/hr), MS mode collision energy 25 V, and MS_1/MS_2 mode collision energy 15 V. Two selective transitions (MS_1>MS_2), 136.9>92.9 for salicylic acid and 140.9>96.9 for the free standard, were used to perform multiple reaction monitoring (MRM) detections. Data were processed by Target-Lynx of MassLynx™ Software, version 4.1 (Waters). The quantification of salicylic acid was corrected by the recovery rate of standard found in each sample.

Results

Transient transformation efficiency

The effects of SA, AIP, PBZ and LA on transient expression in *P. hybrida* and *N. benthamiana* are shown in Fig. 4A. Generally, gene transfer efficiencies were lower in *N. benthamiana* compared to *P. hybrida*. Adding SA in the transformation media showed a severe reduction in transient expression in both plant species. In *P. hybrida*, the inclusion of 10 µM PBZ in the media during transformation resulted in the highest transient transformation efficiency (23.71 ± 1.30), and the effect was significant. For AIP, the explants treated with 10 µM AIP showed the highest transient expression (30.91 ± 5.00), but the effect was not significant. At higher concentrations of PBZ and AIP, transformation efficiency dropped and more severe effects were often observed at 100 µM treatments. In *N. benthamiana,* only 50 µM AIP showed a significant increase in transient expression (23.6 ± 1.67), and no evidence of any stimulatory effect of PBZ was observed. In improving the plant's condition, 10 µM LA resulted in highest transient expression in *P. hybrida* (29.71 ± 2.81) and this was significantly different from other LA concentrations. In *N. benthamiana,* LA did not enhance gene transfer.

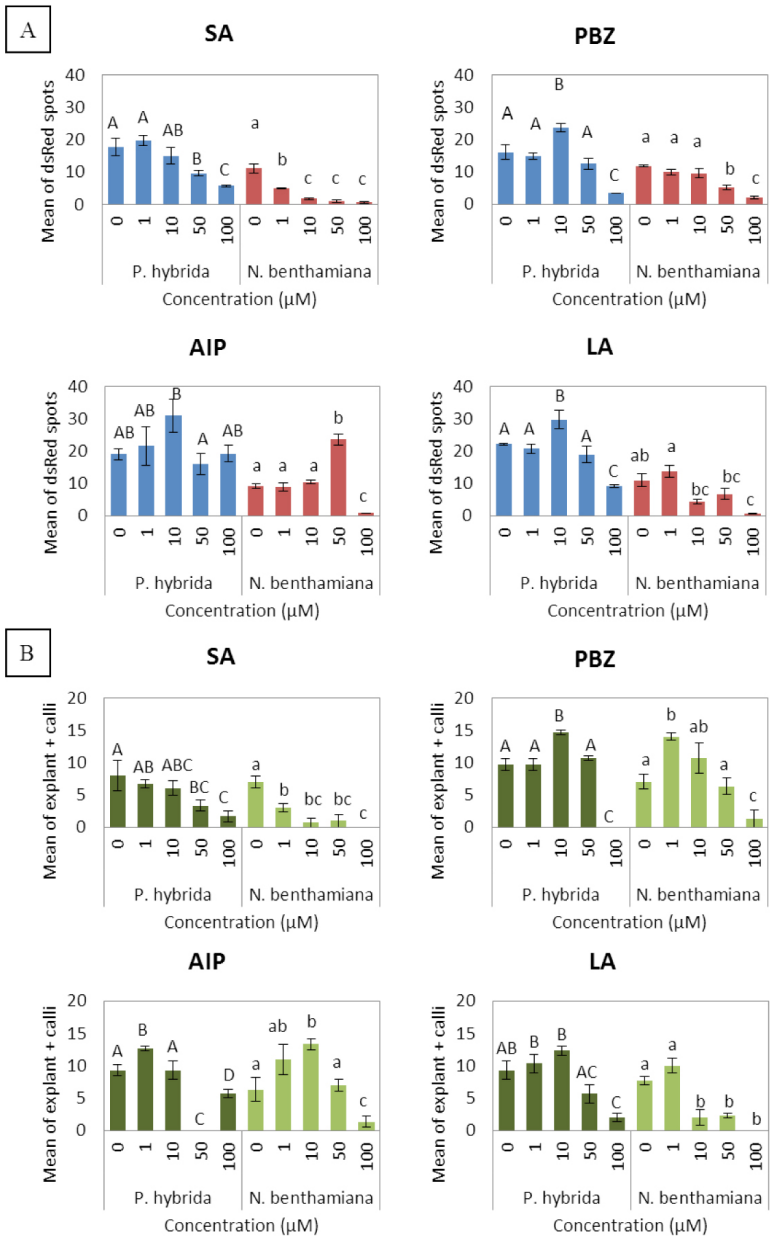

Fig. 4. Effects of different concentrations of SA, PBZ, AIP and LA on (A) transient transformation efficiency and (B) stable transformation efficiency in *P. hybrida* and *N. benthamiana*. Error bars represent the standard error from three replicated plates, each plate containing 15 leaf discs. Values in a column followed by different letters are significantly different at $P<0.05$ according to Duncan's multiple range test.

Stable transformation efficiency

Fig. 4B shows the effect of each compound on stable transformation in both plant species. It was found that adding SA also decreased stable transformation efficiency in both plant species as it did transient expression. 1 µM AIP significantly increased stable transformation efficiency in *P. hybrida* up to 12.67 ± 0.33 compared to 9.33 ± 0.88 with 0 µM. However, the highest stable transformation efficiency in *P. hybrida* was achieved at 10 µM PBZ (14.67 ± 0.33) and this was significantly different from other PBZ concentrations. In *N. benthamiana*, 10 µM AIP and 1 µM PBZ resulted in the highest stable transformation efficiency up to 13.33 ± 0.88 and 14 ± 0.58, respectively, and the effect was significant. No significant stimulatory effect of LA on stable transformation efficiency was found, but the explants treated with LA at 1 and 10 µM were more green and produced bigger calli than non-treated explants in *P. hybrida* (data not shown).

Confirmation of transformation by rooting assay, dsRed fluorescence and PCR analysis

A total of 64 *P. hybrida* and 89 *N. benthamiana* plants were regenerated using the described direct shoot formation method applying selection on kanamycin. The plants have been selected on the rooting media containing kanamycin for 30 days. All regenerated *P. hybrida* plants were checked under a stereo microscope with UV light (Fig. 3 E-F). Out of 64 *P. hybrida* plants, 55 plants were red and nine were not. The red fluorescing plants were variable in the redness intensity from barely red, red and very red (Fig. 3E-I) indicating that the expression level was variable among the transformants. To further test the transgenic nature of the putative transformants, in addition to the growth on selective medium and red fluorescence, we randomly selected four red-positive plants, four red-negative plants and a non-transformed control to perform PCR. As shown in Fig. 5A, all eight putative transgenic *P. hybrida* plants, both fluorescing as well as non-fluorescing showed the presence of the transgene's fragment of approximately 500bp. For *N. benthamiana*, representative samples of four red-positive plants, five red-negative plants and a control were randomly selected for PCR analysis. It was found that all nine putative transgenic *N. benthamiana* plants contained the expected PCR products (Fig. 5B).

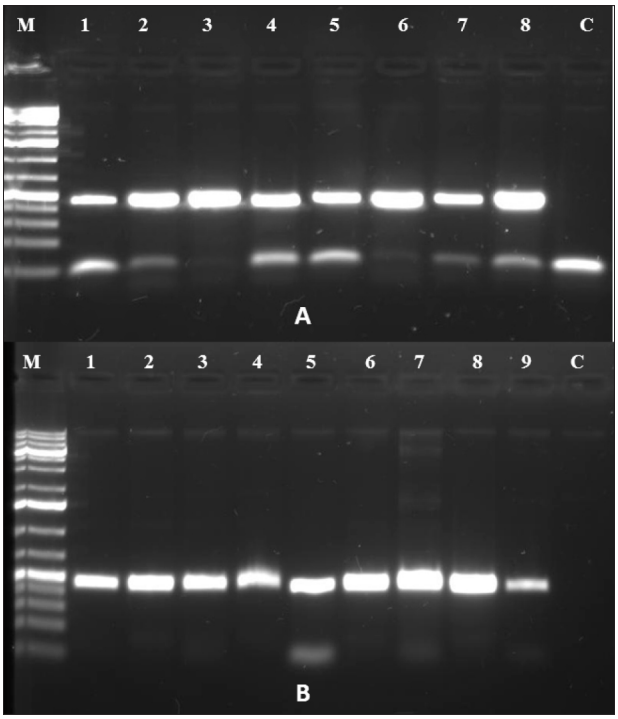

Fig. 5. PCR results of putative transgenic (A) *P. hybrida* plants (B) *N. benthamiana* plants. The expected size of the *nptII* PCR fragment is approximately 500bp; M molecular weight marker with 100bp steps; C non-transformed control. (A) Lanes 1-4 indicated red-positive *P. hybrida* plants; lanes 5-8 red-negative *P. hybrida* plants. (B) Lanes 1-4 indicated red-positive *N. benthamiana* plants; lanes 5-9 red-negative *N. benthamiana* plants.

Free SA level

We measured the free SA level in *P. hybrida* leaf discs supplied with and without exogenous SA (Fig. 6). Inclusion of exogenous 100 µM SA in co-culture + 14 days selection medium significantly increased the free SA level (1359.96 ± 420.45 pg/mg) compared to without SA (261.44 ± 28.41 pg/mg) . The transient transformation efficiencies with SA added were always lower than without SA. Fig. 7 shows the result of measurement of free SA level in *P. hybrida* leaf discs treated with PBZ in co-culture + 14 days selection media. In this experiment, *P. hybrida* produced a basic level of SA approximately at 563.67 pg/mg of fresh weight in response to the *Agrobacterium* infection without PBZ treatment. No reduction in free SA level was observed applying 10 µM PBZ (697.77 ± 19.54 pg/mg), but it was reduced when 50 µM PBZ (427.70 ± 62.22 pg/mg) was employed. The differences of SA level in controls between the two experiments were considerable; therefore comparisons were made only within individual experiments related to its own control. Free SA levels in PBZ-treated leaf discs, however, were not in agreement with transient transformation results, where the highest TTE was observed at 10 µM PBZ, and then dropped at a concentration of 50 µM.

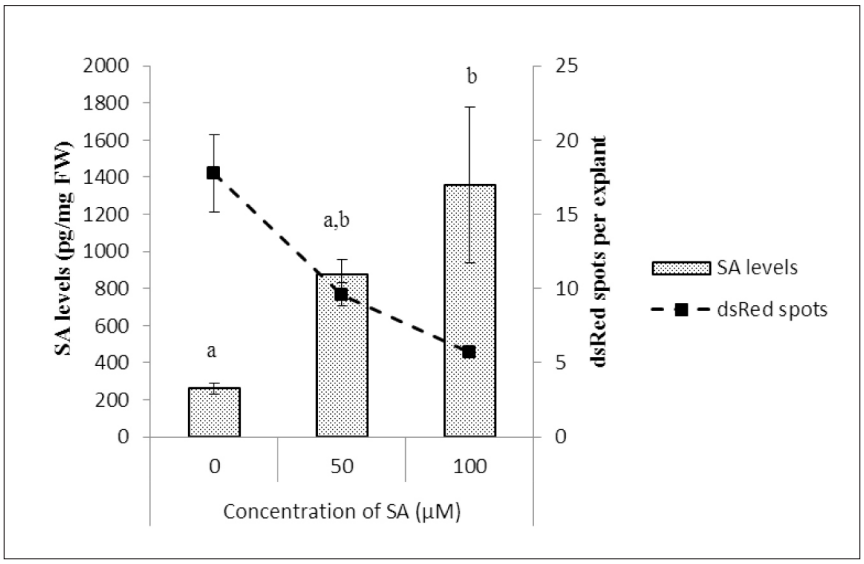

Fig. 6. Comparison between free SA levels and transient transformation efficiency as measured by *dsRed* spots in *P. hybrida* at 0, 50 and 100 µM exogenously applied SA. Treatments with different letters are significantly different for free SA levels (P<0.05).

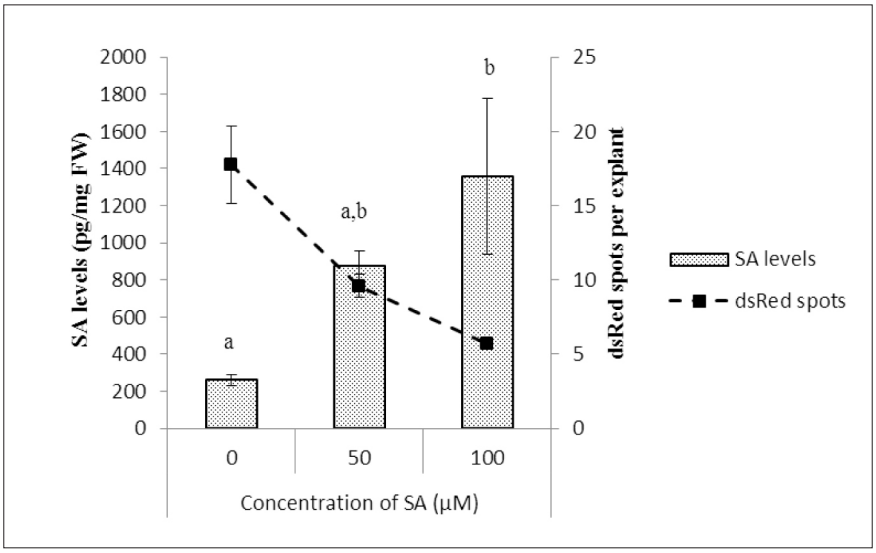

Fig. 7. Comparison between free SA levels and transient transformation efficiency as measured by *dsRed* spots in *P. hybrida* at 0, 10 and 50 µM PBZ. Treatments with different letters are significantly different for free SA level (P<0.05).

Discussion

Agrobacterium-mediated transformation has been previously reported as the most preferred method for plant transformation, but the transformation efficiency can be low and variable among different plant species. The success rate of *Agrobacterium*-mediated transformation highly depends on the plant species, bacterial strain, transformation and regeneration conditions (Godwin et al., 1991; Krens et al., 1988). More so, the efficiency in this transforming method is greatly influenced by a balanced interaction between the two parties involved, the plant and *Agrobacterium.* The plants must be susceptible to *Agrobacterium*, so that the introduced gene can be transferred and integrated into the plant genome. We demonstrated that the *Agrobacterium*-mediated transformation efficiency was reduced by adding SA, providing direct evidence for the role of SA in natural defensive responses against pathogen infection. Blocking SA production via the phenylalanine pathway by inhibitors such as AIP and PBZ at effective concentrations improved the transformation efficiency.

The small phenolic compound, SA, plays an important role in plant defensive responses against biotrophic pathogen infection (An and Mou, 2011; Zhang et al., 2002). We found that adding SA reduced plant transformation efficiency in *P. hybrida* and *N. benthamiana.* On the other hand, blocking the phenylalanine pathway in SA biosynthesis by inhibitors such as AIP and PBZ could indeed promote plant transformation efficiency. In this way, the plant cells become more susceptible to *Agrobacterium* because of a reduction in SA biosynthesis and hence transformation efficiency was increased. The transfer of T-DNA is mediated by virulence (*vir*) genes, which can be induced by various phenolic compounds, and acetosyringone is the most commonly used in *Agrobacterium*-mediated transformation (Cha et al., 2011; Srinivasan and Gothandam, 2016). In this experiment, the inhibitors and acetosyringone were added in both co-cultivation and selection media only for the first 14 days to give an equal chance for the remaining *Agrobacterium* to penetrate and transfer its T-DNA into the explants. The optimum concentrations of SA inhibitors and LA in *P. hybrida* and *N. benthamiana* are presented in Table 1. For AIP, the inclusion of 50 µM AIP in culture media during the transformation process led to enhanced gene transfer in *N. benthamiana.* However, the use of lower concentration (10 µM AIP) was effective in stable transformation efficiency. In *P. hybrida,* only 1 µM AIP showed a positive effect in enhancing stable transformation efficiency. Although the highest transient expression was seen at 10 µM AIP, the effect was not significantly different from that without AIP treatment. This is probably due to variation between the replicates within a concentration being larger than variation between the concentrations. From this experiment, the effective concentration of AIP in enhancing transient transformation was not consistent with the stable transformation. In contrast, the use of 10 µM PBZ in plant transformation was consistently effective in both transient and stable transformations of *P. hybrida*. The effects of AIP and PBZ vary considerably in

different plant species. However, the inhibitors became toxic at a concentration of 50 µM, and caused reduction in transformation efficiency with an exception for AIP treatment in *N. benthamiana* transient transformation. Transformation efficiency further decreased and became detrimental at 100 µM.

Table 1. The optimum concentration of PBZ, AIP and LA showing the highest transformation efficiencies in *P. hybrida* and *N. benthamiana*.

	P. hybrida	*N. benthamiana*
Adding PBZ	10 µM PBZ increased TTE*	-
	10 µM PBZ increased STE*	1 µM PBZ increased STE*
Adding AIP	-	50 µM AIP increased TTE*
	1 µM AIP increased STE*	10 µM AIP increased STE*
Adding LA	10 µM LA increased TTE*	-
	-	-

*represents a significant difference between treatment and without treatment ($p<0.05$).

To further confirm our hypothesis, we determined free SA levels both after SA treatment as well as after treatment with one of the inhibitors. Since PBZ showed the most efficient and consistent effects in enhancing *Agrobacterium*-mediated transformation efficiencies in *P. hybrida,* we measured the changes in free SA levels in inoculated leaf discs treated with and without PBZ. Leaf discs were collected after the 14 days of treatment. Observing that the controls in two independent experiments (Figs. 6 and 7) were variable, hence treatments were only compared to their own control. Our data showed that free SA levels in the leaf discs after exposure to SA were significantly increased and as stated earlier transformation efficiency was decreased, so in agreement with our hypothesis. Free SA levels in leaf discs at 14 days after treatment with an inhibitor was only reduced when 50 µM PBZ was employed and no reduction was seen at 10 µM PBZ. This, however, not seem to be in agreement with the transient transformation results. Endogenous free SA maybe produced via alternative biosynthesis pathway (i.e from isochorismate), or freed by hydrolysis of SA conjugates (see Fig. 1; An and Mou, 2011; Chen et al., 2009; Vlot et al., 2009) during the 14 days exposure. It could be that when BA2H is inhibited at a relatively low level by PBZ, this reduction in PAL-induced SA level can be compensated by the free SA from other sources at the time it was measured. When SA biosynthesis is lowered, SA can be released gradually from its conjugated forms and maintains prolonged systemic acquired resistance (Lee et al., 1995; Pierpoint, 1994). As a large part of SA is in the conjugates (Alvarez, 2000), free SA generated from its conjugates could be responsible for the small difference in levels or to

none at all, that we observed at day 14. Also, it was demonstrated that conjugated forms of SA have a very weak contribution to disease resistance (Panina et al., 2005; Vasyukova and Ozeretskovskaya, 2007). The presence of free SA released from SA conjugates is unlikely to be involved in plant defence against pathogen. Biochemical studies using radiolabelling revealed that SA was formed rather rapidly from *o*-coumarate or/and benzoate produced by PAL, when plants were infected by pathogens (León et al., 1995; Meuwly et al., 1995), showing that PAL-induced SA has specificity in a quick plant defence responses against pathogen attacks. Taken together, the rapid effect of SA and PBZ treatment on free SA levels and gene transfer efficiency, and the late moment of determining free SA levels in our setup could explain the seeming discrepancy between measured transformation efficiency and free SA levels after PBZ treatment.

To assess the transgenic nature of the regenerated plants that have been selected by the rooting selection assay for 30 days, we performed PCR analysis using primers for the *nptII* gene. A DNA fragment of approximately 500bp was amplified from all the selected red-positive and red-negative plants, which indicated a successful transfer of the kanamycin selection gene (*nptII*) into the plant. Together with the variable redness intensity and red-negative results seen among the regenerated plants, it is suggested that the expression of the non-selective reporter gene (*dsRed*) could be either reduced or silenced. It is very unlikely that the gene is truncated or partly integrated since the reporter gene is located in between the right border and *nptII* gene (kanamycin marker), which is located close to the left border in the construct used (Fig. 2). The transfer of T-DNA to plant cells by *Agrobacterium* is thought to start at the right border (Zambryski, 1988; 1992; Zupan and Zambryski, 1995) and complete T-DNA insertion is often identified by using constructs with selectable markers located near the left border (Becker et al., 1992). Therefore, plant cells harbouring the *nptII* gene are very likely to also have the *dsRed* reporter gene in their DNA. Nevertheless, to confirm the integration of the *nptII* and *dsRed* genes into the plant genome, a Southern blot analysis or inverted PCR on the digested plant DNA should be performed.

Browning and necrosis of the infected plant parts is likely to occur, also resulting in low transformation efficiency. The infected plant parts suffer oxidative stresses (Dan et al., 2009; Pitzschke, 2013; Zhang et al., 2002), which is mainly caused by plant response to *Agrobacterium* (Dan et al., 2015). Therefore, improving the plant's condition to cope with such stresses can be considered essential to enhance transformation efficiency. The underlying causes of tissue browning are the accumulation of reactive oxygen species (ROS), phenolic compounds and stimulation of pathogenesis-related (PR) genes in the inoculated leaf discs as a natural defence response, especially against pathogen infection (Pitzschke, 2013; Vlot et al., 2009). LA has been shown to play an important role in plant cells protection

against ROS and is claimed as a general *Agrobacterium*-mediated transformation enhancer (Dan et al., 2009; 2015). Previous studies demonstrated that the use of 5-10 µM of LA improved transformation efficiency in tomato (Dan et al., 2009). However, transformation of Mexican lime, *Citrus aurantifolia* was improved at 50 µM of LA (Dutt et al., 2011); transformation of *Lilium* was improved with 50 mM of LA (Cáceres et al., 2011). In the present study, our results in *P. hybrida* showed that LA only had a significant positive effect at 10 µM on gene transfer efficiency (transient). The effect on stable integration was not significant compared to the control. In contrast in *N. benthamiana,* both transient and stable transformation efficiencies decreased with increasing concentrations of LA. It is possible that *N. benthamiana* does not produce excessive amounts of phenylpropanoid compounds giving rise to oxidative stress, making this species susceptible to a wide variety of plant-pathogenic agents including *Agrobacterium* (Goodin et al., 2008). Hence, improving the condition of *N. benthamiana* plants to become more receptive to *Agrobacterium* by adding LA has no beneficial effect anymore, while in other crops where the excessive formation of phenylpropanoid is hampering *Agrobacterium*-mediated transformation, the use of LA can have a bigger effect. These findings indicate that the response varies between plant species, therefore, it is suggested that LA is a species-dependent enhancer rather than a general transformation enhancer as previously claimed by Dan et al. (2009).

In conclusion, adding SA always decreased the transient and stable transformation efficiencies and increased the free SA level, confirming the role of SA in the plant defence mechanism against pathogen infection in *P. hybrida* and *N. benthamiana* as dicot model systems. Our results indicated that blocking SA biosynthesis through the phenylalanine pathway could promote gene transfer, but the optimum concentrations of inhibitors were variable among different species. Indications have been found that AIP and PBZ as SA inhibitors and LA as transformation enhancer play a role in enhancing *Agrobacterium*-mediated transformation efficiency in dicots and possibly in monocots that were recalcitrant to transformation with *Agrobacterium*.

CHAPTER 3

The *ROSEA1* and *DELILA* transcription factors control anthocyanin biosynthesis in *Nicotiana benthamiana* and *Lilium* flowers

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Abstract

The activity of anthocyanin biosynthesis genes is regulated at the transcriptional level, thus manipulation of transcription factors (TFs) is an ideal strategy to alter the expression of multiple target genes. In this study, we investigated the effect of introducing *ROSEA1* (*ROS1*, a MYB-type) and *DELILA* (*DEL*, a bHLH-type) TFs from snapdragon under control of a flower specific promoter*, Floral Binding Protein 1* (*FBP1*) from petunia into *Nicotiana benthamiana* flowers and *Lilium* tepals. The usefulness of the *FBP1* promoter was demonstrated by the generation of purplish flowers in otherwise normal-growing plants of *N. benthamiana*, while the expression by the *35S* promoter led to the development of stunted plants with anthocyanins in all parts. *N. benthamiana* was successfully transformed by *ROS1* alone and by a combination of *ROS1+DEL*. The observed accumulation of delphinidin corresponded to the expression of *NbCHS, NbF3H, NbDFR* and *NbANS*. The effect of *ROS1+DEL* on *Lilium* flower colour was investigated using agroinfiltration. A higher cyanidin accumulation was observed in tepals of the Oriental hybrid lily cultivar 'Perth', resulting in deeper pink colouration at the infiltrated area. Nevertheless, the introduction of *ROS1+DEL* did not produce any phenotypic changes to the white-flowered *L. longiflorum* cultivar 'Lincoln' and the white Oriental hybrid lily cultivar 'Rialto' due to other deficiencies in their anthocyanin biosynthetic pathway. Co-expression of *ROS1+DEL* under control of the *FBP1* promoter together with active structural anthocyanin biosynthetic genes can result in modification of *Lilium* flower colour.

Keywords: Anthocyanin, transcription factors, flower specific promoter, *N. benthamiana, Lilium* spp.

Introduction

The genus *Lilium* (Liliaceae) represents a bulbous monocot consisting of more than 100 species (Comber, 1949; Lighty, 1968; Lim et al., 2008). The hybrids are divided into several groups namely, Longiflorum (L), Oriental (O) and Asiatic (A) hybrids. The Oriental and Asiatic hybrid lilies have been commercially predominant in the ornamental cut flower industry due to their outstanding flower shape, fragrance and large colour variations (Lim and van Tuyl, 2006; Yamagishi and Akagi, 2013). Lilies from the Longiflorum group nowadays have become more desired among growers and consumers because of several positive traits such as beautiful flowers, early flowering, easy to grow and propagate, and vase life quality (Martens et al., 2003). However, they only exist in purely white-flowered cultivars. Plant breeders have developed various flower colours in the Oriental and Asiatic hybrid lilies using interspecific hybridization (Barba-Gonzalez et al., 2004; Lim and van Tuyl, 2006; Yamagishi and Nakatsuka, 2017). A wide interspecific hybridization between *L. longiflorum* and other lilies from the Asiatic group resulted mainly in sterile F1-hybrids, thus hampering its breeding program (Karlov et al., 1999). Genetic modification offers an alternative approach for the introduction of novel flower colour traits into this plant species. This approach has been successfully developed in another commercially important ornamental crops such as roses and carnations (Chandler and Tanaka, 2007; Chandler and Brugliera, 2011).

Flower colour is an important characteristic that determines the commercial value of ornamental crops. Much interest had been placed in ornamental crops that bear flowers exhibiting large colour variations and hues. Anthocyanins are pigments that provide colours in red, purple and blue to plant organs (Tanaka and Ohmiya, 2008). There are three major groups of anthocyanidins present in higher plants, i.e. cyanidin, pelargonidin and delphinidin (Schwinn and Davies, 2004). Coloured *Lilium* flowers contain cyanidin 3-O-β-rutinoside as a major anthocyanin and cyanidin 3-O-β-rutinoside-7-O-β-glucoside as a minor anthocyanin (Nørbæk and Kondo, 1999).

Because the accumulation of anthocyanins during flower development usually, but not always, correlates with the increasing expression of structural genes, the transcription of the structural genes would appear to be the key factor in controlling the production of anthocyanins. Gene expression is mainly controlled at the transcriptional level and is regulated by MBW complexes consisting of members of the *MYB*, basic helix-loop-helix (*bHLH*) and *WD40* transcription factor families (Quattrocchio et al., 1998; Koes et al., 2005). Among these three transcription factors, co-expression of *MYB* and *bHLH* is essential for activation of anthocyanin structural genes (Bovy et al., 2002; Butelli et al., 2008) whereas *WD40* is needed to stabilize the MBW complexes (Hichri et al., 2011). *MYB* has been most extensively studied and was shown to play the most important role in regulating anthocyanin production, as it can bind directly to the target genes (Hichri et al., 2011). In several plant species, the expression of the *MYB* alone is sufficient to stimulate anthocyanin production, as

was demonstrated e.g. by overexpression of *PAP1* in arabidopsis (Borevitz et al., 2000; Shi and Xie, 2014), anthocyanin1 (*ANT1*) in tomato (Schreiber et al., 2012), *MYB10* in apple, strawberry and potato (Kortstee et al., 2011) and in gerbera (Elomaa et al., 2003). By contrast, expression of the maize *C1* (*MYB)* failed to stimulate anthocyanin accumulation in tomato without the *LC* transcription factor (*bHLH*; Bovy et al., 2002). The results indicated that some plants require both *MYB*- and *bHLH*-type transcription factors to activate anthocyanin biosynthesis, while some others may require only one of the transcription factors.

Different transcription factors can regulate the expression of different sets of the structural genes (Quattrocchio et al., 1993). This means that selection of transcription factors from suitable sources depending on crops might be an important factor in determining successful genetic engineering strategies. *ROSEA1* (*ROS1*) and *DELILA* (*DEL*) transcription factors from snapdragon interact to control anthocyanin accumulation in flowers (Martin et al., 1991). Expression of *ROS1* affects anthocyanin accumulation in both the corolla lobes and tubes, whereas *DEL* is only effective in the corolla tube (Schwinn et al., 2006). The expression of the late structural genes including *flavanone 3-hydroxylase* (*F3H*), *dihydroflavonol 4-reductase* (*DFR*), *anthocyanidin synthase* (*ANS*) and *UDP-glucose 3-O-flavonoid transferase* (*UFGT*) are thought to be regulated by *DEL* (Martin et al., 1991). On the other hand, *ROS1* controls the expression of *F3H*, *flavonol synthase* (*FLS*), *flavonoid 3'-hydroxylase* (*F3'H*), *DFR, leucoanthocyanidin dioxygenase* (*LDOX*) and *UFGT* (Schwinn et al., 2006). In most cases, *DEL* is often required to support the function of *ROS1* (Butelli et al., 2008; Maligeppagol et al., 2013; Outchkourov et al., 2014), although overexpression of *DEL* alone has been reported to induce anthocyanin accumulation in tobacco (Naing et al., 2017).

Floral Binding Protein 1 (*FBP1*) is a flower-specific promoter which is expressed in the petals and stamens of *Petunia hybrida* (Angenent et al., 1993). The *FBP1* promoter has been used to study flower senescence in carnation and herbicide resistance in lily (Bovy et al., 1999; Benedito et al., 2005). The observation that *FBP1* was active in petals and stamens from the primordia stage to mature flowers of carnation indicated that this promoter is still expressed in the later stages of flower development when anthocyanin is produced.

This study investigated the effect of introducing *ROS1* and *DEL* separately or combined on inducing anthocyanin biosynthesis in the flowers of *Nicotiana benthamiana* and *Lilium* spp.. A method for efficient transient transformation by agroinfiltration for lily tepals was developed and optimized. Our study demonstrates that the introduction of *ROS1* alone and a combination of *ROS1+DEL* activated delphinidin biosynthesis in the flowers of *N. benthamiana*. Purplish flowers generated from the phenotypically-normal transgenic plants showed the efficacy of the *FBP1* promoter in driving tissue-specific gene expression, while the use of the constitutive cauliflower mosaic virus (CaMV) *35S* promoter led to the generation of stunted plants with anthocyanin in all parts of the plants. Cyanidin accumulated

in agroinfiltrated pink tepals of the Oriental hybrid lily cultivar 'Perth' showing darker colouration at the infiltrated area. Unfortunately, *ROS1+DEL* failed to induce anthocyanin biosynthesis in the white tepals of the Oriental hybrid lily cultivar 'Rialto' and of *L. longiflorum* cultivar 'Lincoln' indicating more deficiencies in the pathway of those particular genotypes, which warrants further investigation. The results of this study are a first confirmatory step in the genetic modification of flower colour in monocotyledonous plants.

Materials and methods

Plant materials

N. benthamiana was sown in pots (10.5 cm diameter) in the greenhouse. The greenhouse temperature was maintained at around 21˚C during the day and 19˚C during the night (16/8 h photoperiod). The relative humidity was set at 70%. The soil mixture used in this study was composed of 5% of Swedish sphagnum peat, 41% of grinding clay granules, 5% of garden peat, 4% of beam structure, 33% of steamed compost and 12% of PG-Mix 15-10-20. The bulbs of Oriental hybrid lily cultivar 'Perth' (pink tepals) and 'Rialto' (white tepals) and *L. longiflorum* cultivar 'Lincoln' (white tepals) were purchased from De Jong Lelies Holland BV (Andijk, The Netherlands) and were stored at -1˚C. The bulbs were transferred to 4˚C for 24h before planting in crates (30 cm x 50 cm, 6 bulbs per crate) filled with commercial jiffy substrate (Jiffy Products International B.V., Moerdijk, The Netherlands). The plants were grown in the same greenhouse conditions as mentioned for *N. benthamiana*.

Construction of expression vectors

Total gDNA of *Petunia hybrida* was isolated using Dneasy® Plant Mini Kit (250) Cat. No. 69106 according to the manufacturer's instruction. The flower specific promoter, *FBP1* was amplified by PCR using genomic DNA of *P. hybrida*, and primers were extended with restriction sites to accommodate the directional cloning as DNA insert. Primers used for *FBP1* cloning are given in Table S1. The PCR product of *FBP1* was gel purified and cloned into pENTR/D-TOPO vector (Invitrogen). Fragments of *ROS1-t35S* (NcoI-KpnI) and *DEL-t35S* (NcoI-KasI) were digested from pJAM1890 (or known as *35S:ROS1+DEL* in this study; Wei et al., 2017) using restriction enzymes. The plasmid DNA of pJAM1890 was provided by Professor Cathie Martin (John Innes Centre, UK). All the digestions were performed at 37˚C in a water bath for 1.5 hours. Complete digestion products were separated on agarose gel and the fragments were gel purified. Gel purification was performed using Zymoclean gel extraction kit following the protocol given by the manufacturer (Zymo Research Corp., USA). The *ROS1-t35S* fragment was ligated into the NcoI-KpnI site of pENTR/D-TOPO:*FBP1* to yield pENTR/D-TOPO:*FBP1-ROS1*-t35S. The *DEL-t35S* fragment was ligated into the NcoI-KasI site of pENTR/D-TOPO:*FBP1* to yield pENTR/D-TOPO:*FBP1-DEL-t35S*. Ligation was performed using T4 ligase (Thermo Fisher Scientific) and reaction

was incubated overnight at 16˚C. The resultant pENTR/D-TOPO:*FBP1-ROS1*-t35S was further digested with PacI and SbfI, and this fragment was inserted into the PacI-SbfI site of pBinPLUS (van Engelen et al., 1995) to yield *FBP1*:*ROS1* construct. The pENTR/D-TOPO:*FBP1-DEL*-t35S was digested with KpnI and AscI, and this fragment was inserted into the KpnI-AscI site of pBinPLUS to yield *FBP1*:*DEL* construct. Next, *FBP1*:*ROS1* and *FBP1*:*DEL* were combined into a single cloning vector. The pENTR/D-TOPO:*FBP1-DEL*-t35S was digested with KpnI and KasI, and the isolated fragment subsequently inserted into the KpnI-KasI site of pENTR/D-TOPO:*FBP1-ROS1*-t35S to yield pENTR/D-TOPO:*FBP1-ROS1*-t35S-*FBP1-DEL*-t35S. The plasmid obtained was further digested with PacI and SbfI, and the fragment was inserted into PacI-SbfI site of pBinPLUS to yield *FBP1*:*ROS1+DEL* construct. Finally, after sequence verification, the resultant plasmids obtained were introduced into *A. tumefaciens* strain AGL0 (Lazo et al., 1991).

Agrobacterium-mediated transformation in _N. benthamiana_

The *Agrobacterium* culture in liquid Luria-Bertani broth (LB) medium with 50 mg/L kanamycin and 50 mg/L rifampicin was grown overnight at 28°C, 150 rpm in Innova 4000 incubator shaker (New Brunswick Scientific, UK)) to an OD_{600} ± 1.0. The *Agrobacterium* were then collected by centrifugation Heraeus Multifuge 3S (Kendo Laboratory Products, Germany) at 3000 rpm for 15 min., and resuspended in liquid callus inducing medium (CIM) (4.4 g/L MS, 30 g/L sucrose, 0.89 µM BAP, 5.37 µM NAA, pH adjusted to 5.8, with addition of 100 µM acetosyringone after autoclaving) to an OD600 ± 0.1. *N. benthamiana* leaf discs of about 0.5-1 cm^2 were cut excluding the leaf margin and midrib. The discs were placed upside down and floating in a Petri dish containing 5 mL of *Agrobacterium* suspension. The petri dishes were then incubated in the dark at 24-26˚C for 3 days co-cultivation. After that, the discs were blotted dry with sterile filter paper before placing them upside up to petri dishes containing shoot inducing medium (SIM) (4.4 g/L MS, 30 g/L sucrose, 4.44 µM BAP, 0.54 µM NAA, 8 g/L agar, pH adjusted to 5.8, with addition of 500 mg/L cefotaxime and 50 mg/L kanamycin after autoclaving) for selection. The regenerated shoots were then transferred to rooting media consisting of 4.4 g/L MS, 30 g/L sucrose, 0.29 µM indole acetic acid (IAA), 8 g/L agar and 50 mg/L kanamycin. pH of all media was adjusted to 5.8. Only one shoot per explant was transferred to rooting media, in this ensuring that each regenerated plant represented an independent transformation event.

Optimization of agroinfiltration in lily tepals

First, we optimized an agroinfiltration method modified from previous reports (Yasmin and Debener, 2010; Pinthong et al., 2014; Suzuki et al., 2015; Yamagishi, 2016) using *Agrobacterium* strain AGL0 carrying binary vector pCAMBIA1301+*GUS* (CAMBIA, Australia). Several steps of the optimization experiments were conducted to evaluate

the effects of various conditions on transient transformation in the infiltrated tepals of *L. longiflorum* cultivar 'Lincoln' as a host plant.

A single colony of *Agrobacterium* was inoculated in 10 mL liquid Luria-Bertani broth (LB) medium containing 50 mg/L kanamycin and 50 mg/L rifampicin and grown overnight at 28°C, 150 rpm in Innova 4000 shaker (New Brunswick Scientific, UK). As many of the AGL0 strains grew slowly and to ensure reaching an OD_{600} of approximately 1.0, the culture was kept in an exponential phase of growth, by diluting it 1:10 and growing it for another 24 hours. The following day, *Agrobacterium* was pelleted by centrifugation in Heraeus Multifuge 3S (Kendo Laboratory Products, Germany) at 3000 rpm for 15 min., the supernatant was discarded, and the pellet was washed once with sterile demineralized water. The *Agrobacterium* was centrifuged, the supernatant was discarded, and the pellet was resuspended in two types of infiltration buffer; demineralized water (Yasmin and Debener 2010; Pinthong et al., 2014) or MMA medium (20 g/L sucrose, 5 g/L MS salts (no vitamins), 1.95 g/L MES) (Suzuki et al., 2015; Yamagishi, 2016). Both water and MMA medium were supplemented with 100 µM acetosyringone, the pH was adjusted to 5.6 with NaOH, and both were filter-sterilized. In addition, the effect of specific additives for enhancing gene transfer and transformation efficiency was also evaluated. 2-Aminoindan-2-phosphonic acid (AIP), paclobutrazol (PBZ) and lipoic acid (LA), all at a final concentration of 10 µM were added individually to the infiltration buffer (Hasan Nudin et al., 2015). To optimize the bacterial density, the *Agrobacterium* suspension was adjusted to OD_{600} 0.3 (Suzuki et al., 2015; Yamagishi, 2016) or OD_{600} 1.0 (Yasmin and Debener, 2010; Pinthong et al., 2014), and then incubated at room temperature for 1-3 hours for acclimatization. The middle part of the outer tepals of an intact and closed flower bud were pricked by a sterile syringe-needle to facilitate penetration, and subsequently infiltrated with 0.5 mL of *Agrobacterium* suspensions using a 1 mL needleless syringe. The flower buds at the flower developmental stage 4 (i.e. closed flower buds just before the flowers open) were chosen for agroinfiltration.

To optimize the co-cultivation condition, the infiltrated plants were kept in a greenhouse with 16/8 h light-dark photoperiod or in the dark, where the plants were covered with a carton box. The temperature was set at 21˚C and relative humidity at 60%. The infiltrated tepals were harvested at two time points, days 6 and 9 after the infiltration. The samples were then assayed for *GUS* expression. For each condition, three closed flower buds per plant were infiltrated, and the experiments were repeated three times. Finally, the agroinfiltration method used for modification of flower colour was selected based on the optimized protocol concluded from the experiments above.

Histochemical assay

The histochemical *GUS* assay was performed according to (Jefferson, 1987). Briefly, the tepals were incubated in staining solution overnight at 37˚C, and then decolourized using

80% ethanol. Several ethanol changes were required, incubating 12 hours between each wash. Transient *GUS* expression levels were visually measured using a scale rated from 0 to 4, indicating no expression to very high expression (Fig. 1). The scales were determined based on the infiltrated area having *GUS* expression (Dan et al., 2015).

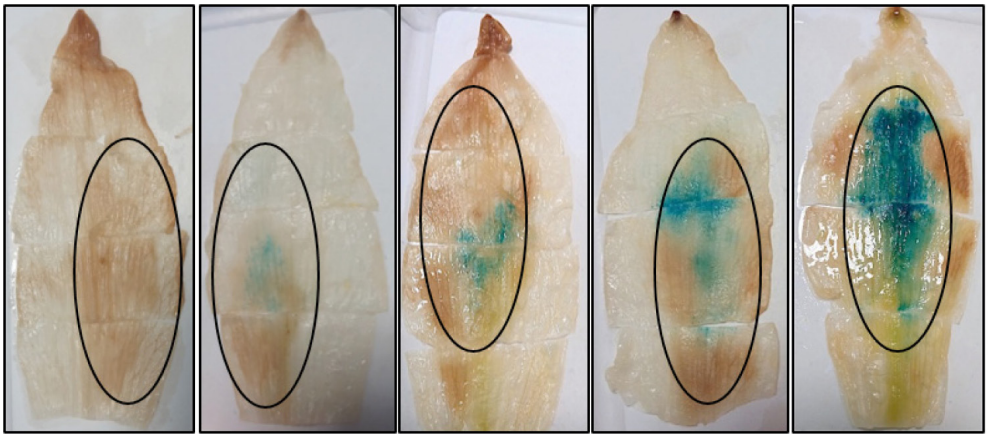

Fig. 1. Expression level scales as used in the *GUS* histochemical assay on lily flower tepals. 0 = no *GUS* expression on infiltrated area; 1 = less than 30% of infiltrated area having *GUS* expression; 2 = 30-50% of infiltrated area having *GUS* expression; 3 = 50-70% of infiltrated area having *GUS* expression; and 4 = 70-100% of infiltrated area having *GUS* expression.

Determination of anthocyanin contents

Anthocyanin and related compounds were extracted from tepals (100 mg dry weight) with 5 ml of extraction solvent containing 1% (v/v) hydrochloric acid (HCl) and 0.1% (v/v) butyl hydroxyl anisol (BHA) in methanol. Three replicates of each sample were used for extraction. The extraction mixture was sonicated for 15 min in ultrasonic cleaner (VWR International, Leuven, Belgium) followed by shaking at 500 rpm in a shaker type HLC for 15 min at 20˚C, centrifuged in 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 2N HCl was heated in a heating block set for 120 min at 99ºC.

The hydrolysed samples 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, flow rate 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 was performed: 5-28% B at 0-35 min, 28-75% B at 35-37 min, 75% B at 37-40 min, 75-5% B at 40-42 min, 5% at 42-50 min. Chromatograms were acquired at 512 nm (anthocyanin) and 280 nm (dihydrokaempferol and dihydroquercetin or taxifolin). Five concentration levels (0.01, 0.025, 0.05, 0.1 and 0.2 mg/mL) of cyanidin chloride, dihydrokaempferol and dihydroquercetin (taxifolin) were used to make calibration curves. Quantification of anthocyanin and related compounds were performed by correlating the chromatographic peak area with concentrations in accordance with the calibration curve of the corresponding external standard.

RNA isolation and cDNA synthesis

Total RNA was isolated from flowers of *N. benthamiana* and the Oriental hybrid lily cultivar 'Perth' (100 mg fresh weight) using TRIzol reagent (Invitrogen Thermo Fisher Scientific) according to the manufacturer's protocol. RNA was treated with DNaseI (Invitrogen Thermo Fisher Scientific). The reaction mixture containing 80 µL RNA, 9 µL 10X DNaseI reaction buffer and 1 µL DNaseI was incubated at 20˚C for 15 min. 10 µL 25 mM EDTA was added to the reaction mixture which was further incubated at 65˚C for 10 min to stop the reaction. RNA was purified using RNeasy mini kit (Qiagen, Germany) according to the manufacturer's protocol. The quantity and quality of RNA were determined using Nanodrop1000™ and gel electrophoresis. First-strand cDNA was synthesized using Taqman reverse transcription reagent kit (Life Technologies, Applied Biosystems #N8080234) according to the manufacturer's protocol. In short, total reaction mixture (50 µL) consisted of 1 µg RNA, 5 µL 10X Taqman RT buffer, 11 µL 25 mM $MgCl_2$, 10 µL 10 mM dNTP mix, 2.5 µL 50 µM oligo dT, 1 µL 20 U/µL RNase inhibitor, 1.25 µL 50 U/µL MultiScribe reverse transcriptase and milliQ water. The mixture was incubated in a PCR machine at 25˚C for 10 min (annealing of primer), 48˚C for 30 min (extension), 95˚C for 5 min (deactivation of reverse transcriptase) and hold at 10˚C. The final concentration of cDNA synthesized was 20 ng/µL.

Quantitative real-time PCR (qPCR)

To investigate the transcription levels of anthocyanin structural genes and transcription factors in the transgenic *N. benthamiana* and lily 'Perth' flowers, quantitative real-time PCR (qPCR) was performed using CFX96TM real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA). Three biological replicates were measured in duplicate. The final reaction volume (20 µL) contained 1 µL of first-strand cDNA, 10 µL 2X iQ SYBR GREEN super mix (Bio-Rad Laboratories, Inc., Hercules, CA, #172-5006 CUST), 2 µL of each forward and reverse primers (3 µM), and 5 µL milliQ water. Cycling conditions were:

preheating at 95˚C for 3 min, 40 cycles of 95˚C for 15 sec 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 sec. The gene specific primers were designed by Primer3Plus program (http://primer3plus.com/) and some were extracted from literatures (Table S1). *L. hybrida Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* and *N. benthamiana Actin* mRNA were 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 S1. The relative gene expression was determined based on the $2^{-\delta\delta Ct}$ calculation method (Livak and Schmittgen, 2001).

Statistical data analysis

Data was subjected to one-way analysis of variance (ANOVA) and means were compared using Duncan's multiple range test at $P<0.05$. The statistical analysis was performed by GenStat 18th edition.

Results

The use of the *FBP1* promoter in transformations

The usefulness of the *FBP1* promoter in *N. benthamiana* transformation was demonstrated by generation of phenotypically normal transgenic plants bearing purple flowers, whereas using the *35S* promoter resulted in purple callus, which developed into stunted plants with anthocyanin accumulation in all plant parts (Fig. 2). In addition, some normal plants with white flowers, which rooted in kanamycin medium were also obtained from the *35S* promoter transformations. The stunted plants could not be maintained and thus only the normal looking plants with white flowers could be used for later analysis.

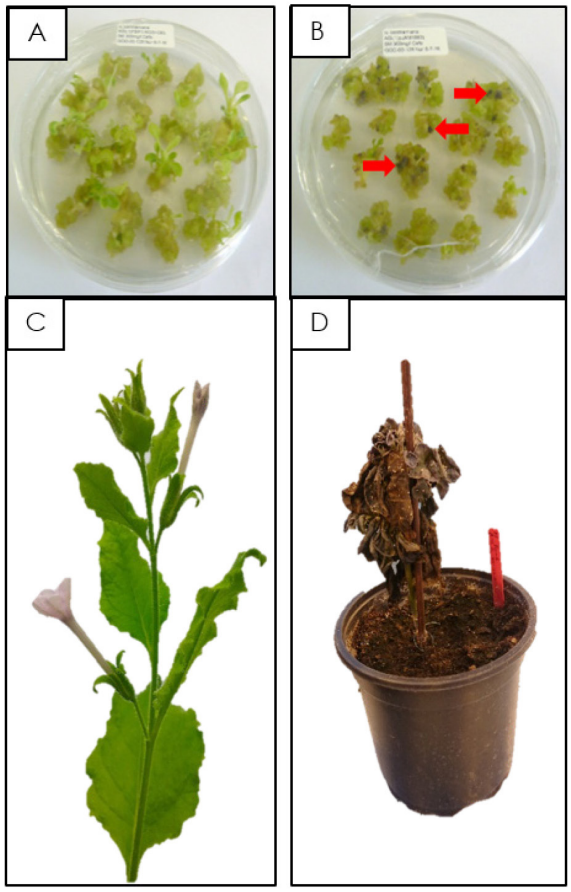

Fig. 2. *N. benthamiana* putative transgenic callus and shoots growing on selection media at 30 days after inoculation, (A) *FBP1:ROS1+DEL* callus cultures; (B) *35S:ROS1+DEL* callus cultures showing dark purple callus (see red arrows). Upon transfer to a greenhouse, anthocyanin accumulated in flowers of *FBP1:ROS1+DEL* expressing plants (C), and in all plant parts of *35S:ROS1+DEL* expressing plants (D).

Optimization of agroinfiltration for lily tepals

To optimize different conditions that could influence the gene transfer by agroinfiltration in lily tepals, *L. longiflorum* cultivar 'Lincoln' was selected as a host genotype due to availability of plants carrying flowers during the time the experiments were conducted. A number of conditions/variations were tested, including the infiltration buffer, bacterial density, light condition, time during co-cultivation and additives to enhance transformation efficiency.

Two different infiltration buffers (sterile demineralized water and MMA medium) and bacterial densities (OD_{600} 0.3 and 1.0) were tested for their effectiveness (Fig. 3). *GUS* histochemical assays indicated that the MMA infiltration buffer with a bacterial density OD_{600} of 1.0 showed positive *GUS* expression at the 0%-30% level. However, no or a few tiny blue spots were observed under the microscope for the *GUS* expression with water as infiltration buffer with both bacterial densities as well as with MMA with a bacterial density OD_{600} of 0.3. Therefore, suspending *Agrobacterium* in MMA buffer at a bacterial density OD_{600} of 1.0 was selected for the next series of experiments.

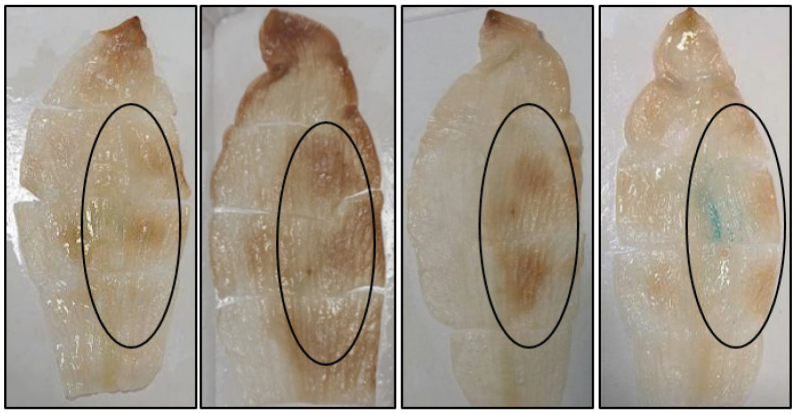

Fig. 3. The effect of different infiltration buffers and bacterial densities on *GUS* expression in *L. longiflorum* cultivar 'Lincoln' tepals determined at 6 days after agroinfiltration.

To determine the optimal light conditions during co-cultivation, the infiltrated flowers were incubated in the dark where the whole plants were covered by a rectangular carton box, or in the normal light condition (16/8h photoperiod) of the greenhouse. For determining the optimal co-cultivation time, samples were harvested at days 6 and 9 after the agroinfiltration (Fig. 4). The *GUS* assay indicated that infiltrated tepals incubated under normal light conditions and harvested at day 6 after the infiltration showed a higher and more intense expression compared to day 9. By contrast, no *GUS* expression was observed in infiltrated tepals incubated in the dark (data not shown).

For the inclusion of additives (Fig. 5), PBZ and AIP at 10 µM in MMA increased the *GUS* expression substantially in lily tepals, i.e. to the highest level, 70%-100%. Inclusion of PBZ in water increased *GUS* expression to 30%-50%, whereas AIP in water only slightly increased *GUS* expression, 0%-30%. The inclusion of 10 µM LA in water showed low *GUS* expression at 0%-30%, and no increase of expression was seen with the inclusion of LA in MMA. Combinations of LA+PBZ or LA+AIP in both infiltration buffers did not show any beneficial effect compared to the effect of PBZ and AIP individually (data not shown).

Therefore, 10 µM PBZ in MMA was selected and used for the agroinfiltration experiments in this study.

Finally, an optimized agroinfiltration method using an *Agrobacterium* suspension in MMA buffer supplemented with 100 µM acetosyringone, 10 µM PBZ, at a bacterial density OD_{600} of 1.0, normal light conditions (16/8h photoperiod) during co-cultivation and the harvesting time at day 6 after the infiltration was selected and used for monitoring transient gene expression of anthocyanin regulatory genes in lily tepals.

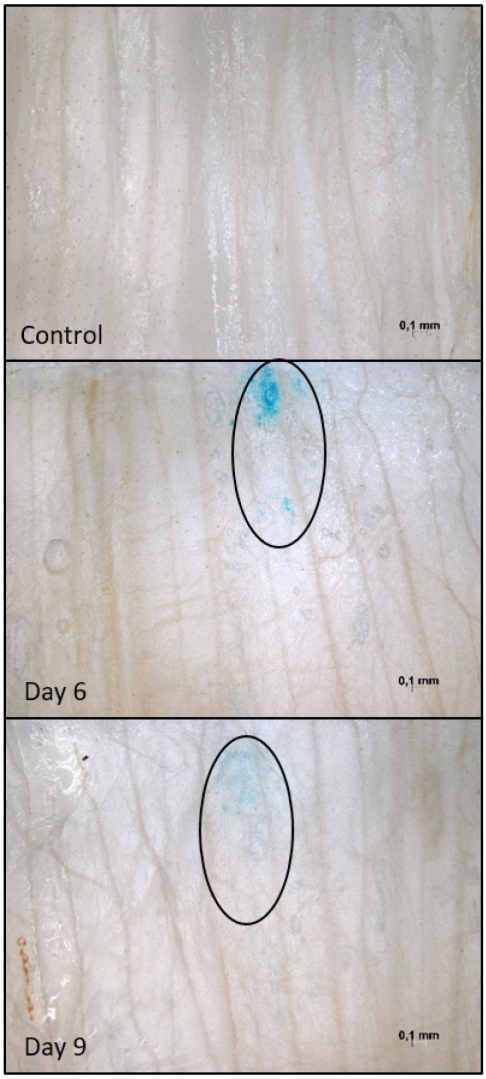

Fig. 4. The effect of co-cultivation time at 6 and 9 days on *GUS* expression in *L. longiflorum* cultivar 'Lincoln' tepals incubated under normal light conditions (16/8h photoperiod). Control is a non-transgenic tepal.

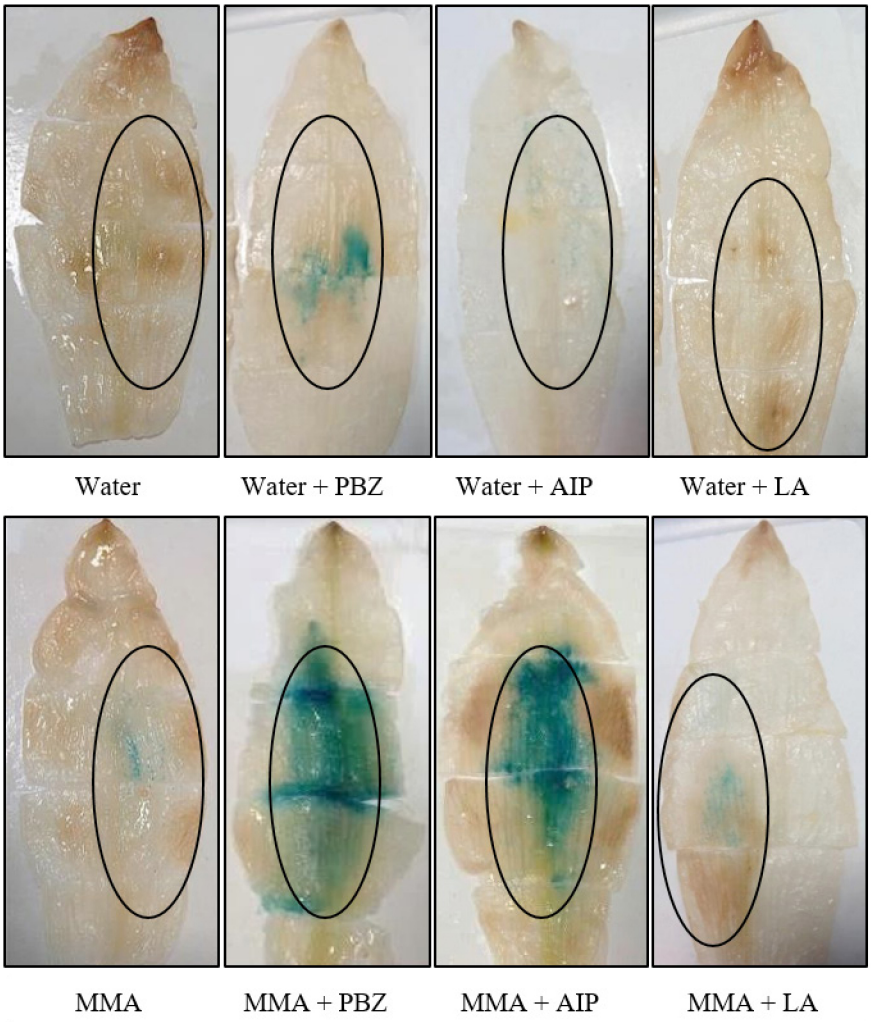

Fig. 5. The effect of the additives on *GUS* expression in *L. longiflorum* cultivar 'Lincoln' tepals harvested at day 6 after agroinfiltration. Each additive at final concentration 10 µM was added into water or MMA as infiltration buffers. PBZ, paclobutrazol; AIP, 2-aminoindan-2-phosphonic acid; and LA, lipoic acid.

The effect of *ROS1* and *DEL* on anthocyanin biosynthesis and gene expression

N. benthamiana

Transgenic plants of *N. benthamiana* expressing *ROS1* or *ROS1+DEL* under the control of *FBP1* promoter produced purple flowers (Fig. 6), due to the presence of delphinidin. The delphinidin content measured by HPLC was compatible with the phenotypic data. Unfortunately, we were unable to determine the anthocyanin content in the

purple flowers expressing *35S:ROS1+DEL* due to insufficient flower material available for analysis at that time. As mentioned earlier, only white flowers from normal looking plants, rooting on selection medium and expressing *35S:ROS1+DEL,* could be used for biochemical analysis. Here, no delphinidin accumulation was observed in the white flowers expressing *35S:ROS1+DEL, FBP1:DEL* and the control wild type. In addition, no significant difference in dihydrokaempferol content was observed between the transgenic plants and control wild type.

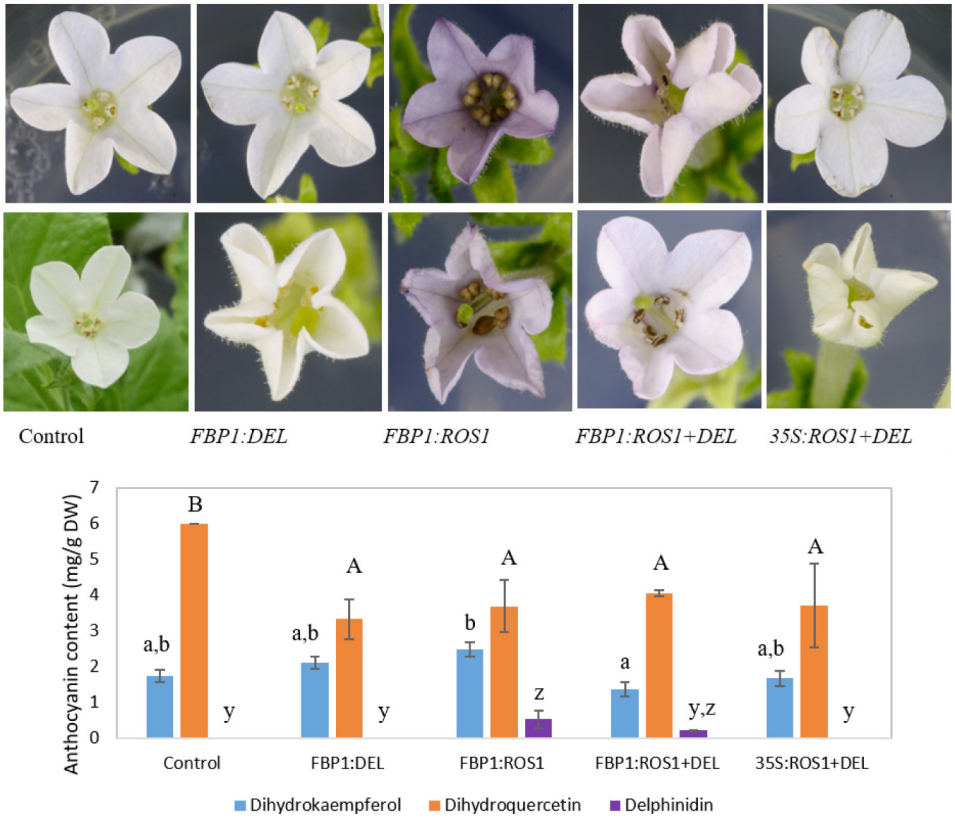

Fig. 6. Phenotypic changes in flower colour (upper panel) and accumulation of delphinidin and dihydroflavonols (lower panel) in the *N. benthamiana* control flowers and in transgenic flowers, carrying *FBP1:DEL, FBP1:ROS1, FBP1:ROS1+DEL* and *35S:ROS1+DEL*.

Next, qPCR was used to examine the expression of anthocyanin structural genes (*NbCHS, NbCHI, NbF3H, NbDFR* and *NbANS*) in the different transgenic flowers. All genes analyzed were expressed and upregulated relative to the wild type white flowers, which differences in expression proved to be related to the constructs being introduced (Fig. 7). The highest expression level was observed in the flowers expressing *FBP1:ROS1*, followed

by *FBP1:ROS1+DEL*. The introduction of *ROS1* alone clearly stimulated the expression of *NbCHS, NbF3H, NbDFR* and *NbANS*, while the expression level of *NbCHI* was generally low compared to the other genes. The late gene, *NbANS,* converting leucodelphinidin into delphinidin was low in both flowers expressing *FBP1:DEL* and *335S:ROS1+DEL*, which might explain the lack of delphinidin accumulation here.

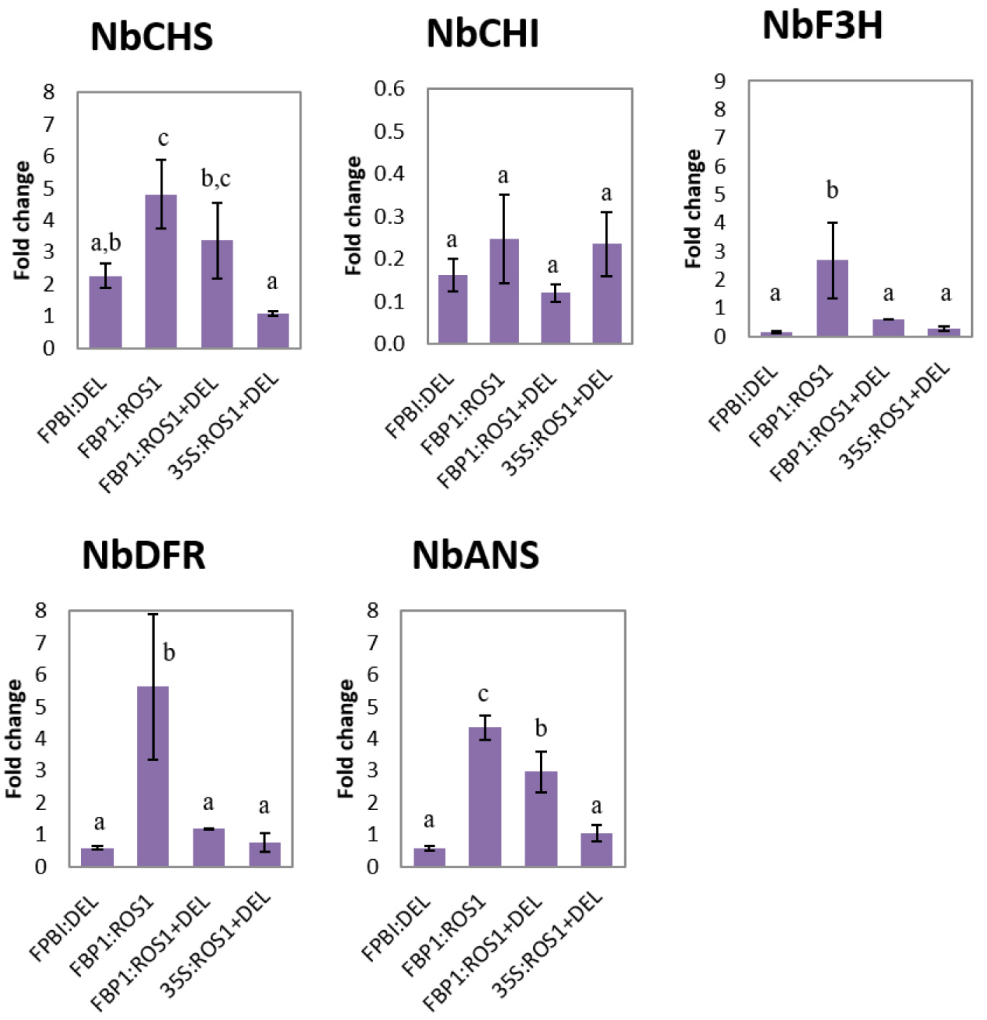

Fig. 7. Relative quantification of *NbCHS, NbCHI, NbF3H, NbDFR* and *NbANS* expression in the flowers of *N. benthamiana* after introduction of *FBP1:DEL, FBP1:ROS1, FBP1:ROS1+DEL* and *35S:ROS1+DEL.* Error bars represent mean standard error calculated from three biological replicates.

Lilium spp.

After the introduction of *ROS1* and *ROS1+DEL* under the control of *FBP1* promoter, anthocyanin accumulated in tepals of the Oriental hybrid lily cultivar 'Perth', showing deeper pink colouration at the infiltrated area compared to the controls with only infiltration buffer and wild type (Fig. 8). The level of cyanidin in tepals expressing *FBP1*:*ROS1+DEL* was comparable to *FBP1*:*ROS1*, which was about 3-fold higher than the wild type control. Introduction of all constructs reduced dihydrokaempferol content in 'Perth' tepals, However, dihydroquercetin could not be detected in all samples. No phenotypic changes were observed in the white tepals of the Oriental hybrid lily cultivar 'Rialto' and *L. longiflorum* cultivar 'Lincoln' (data not shown). Therefore, only tepals of the Oriental hybrid lily cultivar 'Perth' were used for gene expression and anthocyanin measurement analyses. The transcription of eight anthocyanin structural genes (*LhCHSa, LhCHSb, LhCHIa, LhCHIb, LhF3H, LhF3'H, LhDFR* and *LhANS*) in tepals was upregulated after the introduction of all constructs (Fig. 9). Introduction of *FBP1*:*ROS1+DEL* resulted in the highest expression levels of structural genes (*LhCHSa, LhCHSb, LhCHIa, LhCHIb, LhF3'H, LhDFR* and *LhANS*). Introduction of *FBP1*:*ROS1* alone also induced anthocyanin accumulation in tepals, but did not stimulate gene expression to levels higher than that of *FBP1*:*ROS1+DEL*. In contrast, *FBP1*:*DEL* and *35S*:*ROS1+DEL* generally stimulated expression of structural genes to a low level only, consequently, their introduction did not stimulate the accumulation of cyanidin in 'Perth' tepals.

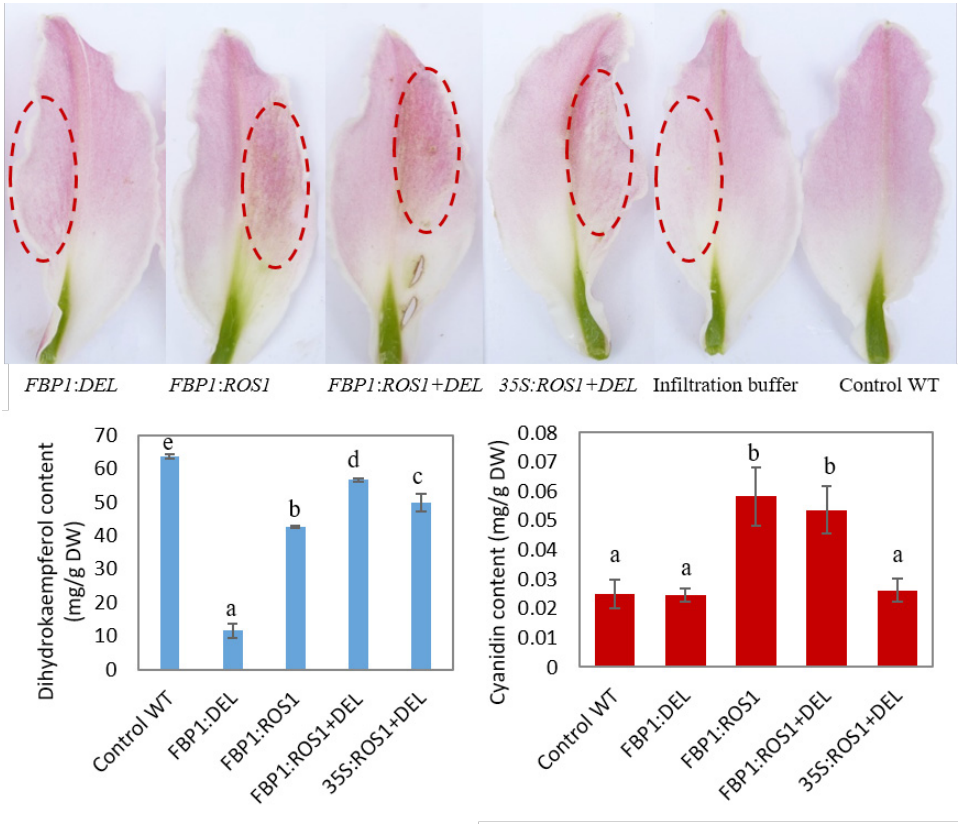

Fig. 8. Phenotypic changes in flower colour at the infiltrated area shown in circle (upper panel) and accumulation of dihydrokaempferol and cyanidin measured at the infiltrated area (lower panel). The flowers are from the Oriental hybrid cultivar 'Perth' introduced by *FBP1:DEL, FBP1:ROS1: FBP1:ROS1+DEL, 35S:ROS1+DEL* or after a mock-infiltration with MMA buffer only, and a control wild type (WT).

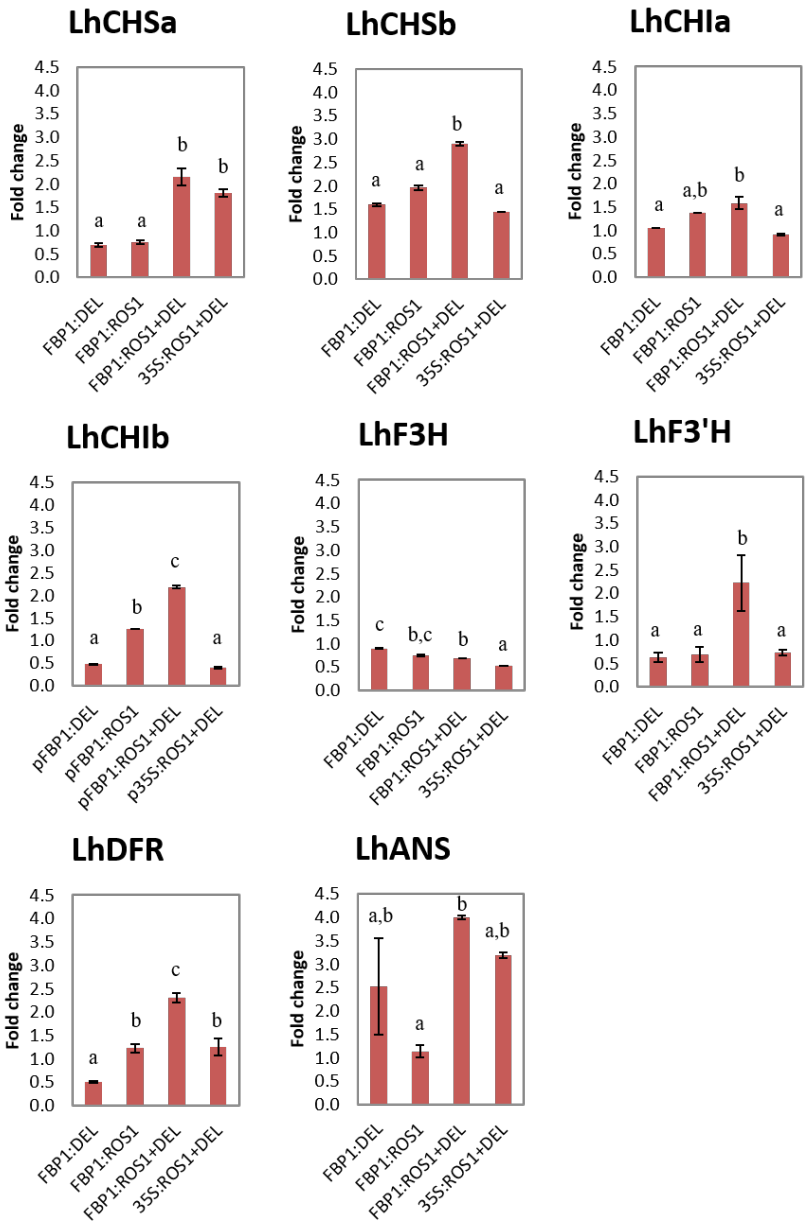

Fig. 9. Relative quantification of *NbCHS, NbCHI, NbF3H, NbDFR* and *NbANS* expression in the tepals of the Oriental hybrid lily cultivar 'Perth' after introduction of *FBP1:DEL, FBP1:ROS1, FBP1:ROS1+DEL* and *35S:ROS1+DEL*. Error bars represent mean standard error calculated from three biological replicates.

Discussion

The cauliflower mosaic virus (CaMV) *35S* is a very common promoter and is widely used for gene expression studies (Chandler and Tanaka, 2007). However, this promoter has not always been effective in yielding a desired result, especially in monocot plants (Tada et al., 1991; Katsumoto et al., 2007), hence requiring a search for a more suitable promoter to increase the expression of genes in a particular tissue. Therefore, we investigated the suitability of *Floral Binding Protein 1* (*FBP1*), a flower-specific promoter from *Petunia hybrida* (Angenent et al., 1993) and the *35S* promoter in controlling *ROS1* and *DEL* transcription factors in a dicot *N. benthamiana* and the monocot lily. We found that the use of the *35S* promoter produced too much anthocyanin in all *N. benthamiana* plant parts, which may have interfered with plant development and finally resulted in a stunted plant. The results obtained indicated that the *35S* promoter is not suitable for colour modification in *N. benthamiana* flowers. In contrast, the transcription factors expressed by the *FBP1* promoter stimulated colour occurrence only in the flowers, in both *N. benthamiana* and the Oriental hybrid lily cultivar 'Perth', indicating that *FBP1* was a suitable promoter for driving tissue-specific expression, particularly in flowers of both dicots and monocots.

Lily is known to be a recalcitrant species to genetic transformation by *Agrobacterium tumefaciens*. In recent years, successful lily transformation protocols have been reported, but the transformation efficiency varied between cultivars and was mostly very low (Wang et al., 2012). In addition, the selection and regeneration of transformed callus into transgenic plantlets may take up to 8 to 10 months (Wang et al., 2012) although faster procedures have been published (Cáceres et al., 2011; Wei et al., 2017). Subsequently, it takes another 18 months from transfer of bulblets to the soil and the greenhouse to first flowering after several months of cold treament. Hence, a transient gene expression system was chosen for lily. In the present study, an agroinfiltration method was first optimized to establish a transient gene expression system for lily flowers and subsequently used to determine the functionality of the *ROS1* and *DEL* transcription factor genes in lily tepals. In this study, the *ROS1* and *DEL* were introduced into lily tepals during the closed flower buds stage 4 (i.e. when the buds are about to open) because most of the structural genes were highly expressed at this stage, as discovered in the Oriental lily cultivar 'Sorbonne' (Yamagishi, 2011). We believe this is the best stage to manipulate the expression of structural genes through the activity of the *ROS1* and *DEL*, thus influencing anthocyanin levels.

The results of the optimization experiments revealed that agroinfiltration in lily tepals was dependent on the infiltration buffer, bacterial density, light condition, time of co-cultivation, and on specific additives for enhancing gene transfer and transient gene expression (Hasan Nudin et al., 2015). It has been reported that sterile demineralized water was used for agroinfiltration of flowers such as roses (Yasmin and Debener, 2010) and orchids (Pinthong et al., 2014). However, our results showed that agroinfiltration of lily tepals works better with

MMA medium (Suzuki et al., 2015; Yamagishi, 2016) compared to sterile demineralized water. Bacterial density has also been considered as a critical factor in determining gene transfer efficiency. For lily tepals, the *GUS* expression was higher at bacterial density OD_{600} 1.0 compared to 0.3. This result is in agreement with previous reports in rose and orchid (Yasmin and Debener, 2010; Pinthong et al., 2014), whereby no significant effect on *GUS* expression was seen at OD_{600} 0.5 up to 4.0, but a weak or no *GUS* expression at OD_{600} below 0.5. In contrast, OD_{600} 0.3 has been commonly used for agroinfiltration in Arabidopsis, lettuce and *N. benthamiana* leaves (Wroblewski et al., 2005; Suzuki et al., 2015). The co-cultivation time had a significant effect on the *GUS* expression level (Yasmin and Debener, 2010). For example in rose, the highest *GUS* expression was detected between days 3 and 7, after which the expression started to decrease significantly. Similarly in lily tepals, higher *GUS* expression was achieved at day 6 compared to day 9. However, unlike rose, we detected *GUS* expression in lily tepals incubated in 16/8h photoperiod and not in the dark. For enhancing gene transfer by *Agrobacterium* or to improve the plant's ability to deal with exposure to *Agrobacterium*, different concentrations of additives and acetosyringone have been tested in several plant species. Acetosyringone is known as an inducer for virulence genes of *Agrobacterium* needed to transfer T-DNA into the host plant (Manfroi et al., 2015). The effect of 100 and 200 µM acetosyringone in agroinfiltration of different rose genotypes was found to be non-significant (Yasmin and Debener 2010), thus 100 µM was chosen in the present study. In agreement with our previous findings in transient and stable transformation of *N. benthamiana* and *P. hybrida* (Hasan Nudin et al., 2015), addition of salicylic acid synthesis inhibitors (PBZ and AIP) at a final concentration of 10 µM in MMA medium strongly improved the *GUS* expression in lily tepals after agroinfiltration.

Anthocyanin biosynthesis in organisms is determined by the expression of multiple structural genes involved in the pathway, and the activation of the genes is mainly controlled at the transcriptional level. Thus, manipulation of the transcription factors is considered an appropriate strategy to alter the expression of structural genes, thus influencing anthocyanin levels. Various studies have examined the overexpression of *bHLH*- and *MYB*-type transcription factors such as *LC/C1* in maize, *DEL/ROS* in snapdragon, and *AN1/AN2* in petunia. Similarly in lilies, *LhMYB12* interacts together with *LhbHLH2* and *LhWD40A* to regulate the transcription of anthocyanin biosynthetic genes (Nakatsuka et al., 2009; Suzuki et al., 2016; Lai et al., 2012; Yamagishi, 2011). According to the *MYB* genes phylogeny (Yamagishi et al., 2012), *AmROS1* and *LhMYB12* were placed together in the same AN2 subgroup, which regulate the expression of late structural genes. On the other hand, *ZmC1* was found to be distantly related to *LhMYB12*, and was placed in the C1 subgroup, which regulates the expression of both early and late structural genes. Therefore, we assumed that in lilies, *ROS1* from snapdragon, which resembled *LhMYB12* in the sequence profile could perform better than *C1* from a monocot gramineous species.

In *N. benthamiana*, the production of delphinidin was stimulated in the flowers after the introduction of *FBP1:ROS1* and *FBP1:ROS1+DEL.* Introduction of *ROS1* alone induced the highest transcription levels of anthocyanin structural genes (*NbCHS, NbF3H, NbDFR* and *NbANS*) in *N. benthamiana.* It is likely that the endogenous *MYB* transcription factor is not functional, and therefore *ROS1* complements through an interaction with an endogenous *DEL*-like transcription factor present in *N. benthamiana*. Flavonoid 3'5' hydroxylase (F3'5'H) is the key enzyme for delphinidin biosynthesis (Qi et al., 2013). Unfortunately, we could not study the expression of the *F3'5'H* gene in transgenic *N. benthamiana*, presumably because the gene specific primers used were based on *N. tabacum* sequences and might lack sufficient homology. However, the generation of delphinidin in the transgenic *N. benthamiana* is a clear indication that a functional *F3'5'H* gene must be present. Opposite to what was found in *N. benthamiana*, it was known already that lily cannot produce delphinidin because of the lack of *F3'5'H* gene (Martens et al., 2003). Thus, delphinidin was not expected in lily tepals without the introduction of an active *F3'5'H* gene itself. In lily 'Perth' (pink tepals), introduction of *FBP1:ROS1* and *FBP1:ROS1+DEL* increased the level of cyanidin, resulting a deeper pink colouration at the infiltrated tepals. The combination of *FBP1:ROS1+DEL* could stimulate the highest transcription of early (*LhCHSa, LhCHSb, LhCHIa, LhCHIb, LhF3'H)* and late structural genes (*LhDFR* and *LhANS*). In contrast, introduction of *FBP1*:*DEL* and *35S*:*ROS1+DEL* failed to stimulate the accumulation of cyanidin in 'Perth' tepals. In the Oriental hybrid lily cultivar 'Sorbonne', the expression of *35S:ROS1+DEL* showed obvious colour change only during the early regeneration stages but the colour faded into normal green transgenic plantlets after rooting (Wei et al., 2017). Taken together the gene expression profile and cyanidin content, it is suggested that the combination of *ROS1+DEL* seems important to increase gene expression levels and anthocyanin production in 'Perth', and the activity of the *FBP1* promoter until the late flowering stage in *N. benthamiana* and 'Perth' was confirmed.

The white tepal colour of the Oriental hybrid lily cultivar 'Rialto' was thought to be caused by a mutation in the *LhMYB12* transcription factor (Yamagishi, 2011). A single W-to-L amino acid substitution was detected in the R2-repeat, which likely disrupted the function of *LhMYB12* (Yamagishi, 2011; Yamagishi et al., 2012). In the wild species *Lilium speciosum,* the white tepal line with red anthers contained a *LsMYB12* gene that was identical to *LhMYB12* of 'Rialto' (Suzuki et al., 2015). The non-functioning of the *LsMYB12* gene was experimentally confirmed in *N. benthamiana* transient expression assays (Suzuki et al., 2015). Moreover, the expression levels of a functional *LhMYB12* gene strongly increased the anthocyanin accumulation in coloured tepals (Yamagishi et al., 2012). Based on these results, it was assumed that the W-to-L mutation in the *Lilium MYB12* gene copy was responsible for the lack of anthocyanin biosynthesis in the white tepals of 'Rialto' and *L. speciosum.* Thus, our aim was to introduce a functional copy of the *ROS1* gene in order to restore

the lack of function of the mutated *LhMYB12* in 'Rialto'. Although on the other hand, the genetic background of the white tepals of *L. longiflorum* cultivar 'Lincoln' is unknown, we also investigated the effect of introducing *ROS1* here. Unfortunately, the introduction of *ROS1+DEL* did not bring about any phenotypic changes to the white flowered 'Rialto' and 'Lincoln'. It could be speculated that some structural gene(s) involved in the anthocyanin biosynthtic pathway in these two cultivars is/are lacking or mutated. Further genetic research into the background behind the white colouration in 'Rialto' and 'Lincoln' will be addressed in our future research.

As a conclusion, *FBP1* from petunia is proven to be a suitable promoter for the expression of structural genes responsible for anthocyanin biosynthesis in the flowers, in a dicot *N. benthamiana* and the monocot lily. An efficient agroinfiltration method for transient gene expression was successfully established for floral tissues in lily. Introduction of *ROS1* alone and a combination of *ROS1+DEL* activated delphinidin as well as cyanidin biosynthesis in the flowers of *N. benthamiana* and the Oriental hybrid lily cultivar 'Perth', respectively. Unfortunately, *ROS1+DEL* failed to induce anthocyanin biosynthesis in the white tepals of the Oriental hybrid lily cultivar 'Rialto' and *L. longiflorum* cultivar 'Lincoln' indicating more deficiencies in the pathway, which warrants further investigation.

Table S1. List of primers used in this study and primer efficiency for qPCR analysis.

Primer	Forward primer 5'-3'	Reverse primer 5'-3'	Reference	Primer efficiency
Primers used for cloning and sequencing				
FBP1-1	CACCTGCAGGAGAAGTA ATAATAATGATACTTCT	TTAATTAAGGCGCCGAC TGGTACCACTGCCATGG TTTCCCTTTCTCTTGTTG GACTAT	This study	-
FBP1-2	CACCGGTACCGAGAAGT AATAATAATGATACTTCT	GGCGCCCAGTCCATGGT TTCCCTTTCTCTTGTTGG ACTAT	This study	-
DEL	CGTAAGACGACTAAGTTTGG	GCCATTTCCCTTCACCA TAC	This study	-
ROS1	CATTCAGTGGTGGAGTAAGT	TGCAACTGAATTGGAC CAGA	This study	-
M13	GTAAAACGACGGCCAG	CAGGAAACAGCTATGAC	This study	-
Primers used for qPCR				
NbCHS	ATAGTTTGGTTGGGCAAGCC	AGACAAGCTGGAACAA AGGC	This study	1.992
NtCHI	CCCAATGGTGCAACTGTAA AGG	TGCCAAGCATGCTCTTC TTC	This study	1.972
NtF3H	TGCATGAAAACCGGTTCGTC	TCGGCCCTACGTGATTT GATAC	This study	1.970
NbDFR	ATTCATCTGCGCATCCCATC	ACCACAGGCAAGTCCT TATCG	This study	1.963
NbANS	TCCTCCACAATATGGTGCCTG	GGGTGTCCCCAATATGCATG	This study	1.976
NbActin	TGCAAAGACCAGCTCTTCTG	ATTCCTGCAGCTTCCATTCC	This study	1.943
LhCHSa	TGGGACTCACCTTCCATCTC	CATGTTTCCGTACTCGCTCA	This study	1.915
LhCHSb	CTGAAGCTGGCGCTGGAC AAAAAG	GGTAGTGATCGGAATGCTG TGAAGA	Yamagishi 2011	1.846
LhCHIa	TCCATCCTCTTCACCCAGTC	CCTTGAGAAGCTCGGAA ATG	Suzuki et al. 2015	1.882
LhCHIb	GCGGTCGATAAGTACGAGGA	TCCCACCCAAATACCAC TTC	This study	1.905
LhF3H	TGCCTTTGTTGTCAATCTCG	GCATCCAAAACTGGCTT CTC	This study	1.947
LsF3'H	ACGCACGACACAAACTTCAG	CGAGTGCTTTCTGGGAA AAG	Suzuki et al. 2015	1.922
LhDFR	ATATGCCATCCCCCAAAAGT	GCAACCCAAATCCAGTT CAT	Yamagishi 2011	1.924
LhANS	GGGGGAATGGATGACCTACT	GTTGGTGAGGAGGAAG GTGA	Suzuki et al. 2015	1.843
LhGAPDH	CTACTGTGCACGCCATCACT	ACACATCGACAGTGGGA ACA	This study	1.957

The restriction sites are underlined: SbfI: CCTGCAGG, NcoI: CCATGG, KpnI: GGTACC, KasI: GGCGCC, PacI: TTAATTAA. Nb, Nt, Lh and Ls indicate the primers were designed based on the *N. benthamiana, N. tabacum, L. hybrida* and *L. speciosum* sequences, respectively.

CHAPTER 4

Molecular mechanisms regulating anthocyanin biosynthesis during flower development in *Lilium* spp.

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Abstract

Sterility of hybrids produced from interspecific hybridization in lilies has proven to be a great limitation in the breeding program, especially for *Lilium longiflorum,* in which only white-flowered cultivars exist. Because modification or enrichment of flower colour in *L. longiflorum* by conventional breeding has not succeeded so far, we investigated whether genetic modification could be used as an alternative to obtain novel colours in this species. For this, we need to understand what determines white colouration in *L. longiflorum* and other species and identify the molecular mechanisms regulating flower colour, especially those involved in the anthocyanin biosynthesis pathway. In this study, we determined the presence and the expression of eight structural genes (*CHSa, CHSb, CHIa, CHIb, F3H, F3'H, DFR, ANS*) and three transcription factor genes (*MYB12, MYB15, bHLH2*) during five 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, we determined the presence of anthocyanins and related compounds in flower tissues. The white colour of 'Lincoln' flowers is likely caused by the absence of two structural genes (*F3'H* and *DFR*) and one transcription factor gene (*bHLH2*). In 'Rialto', *DFR* is present but not expressed thus causing its white flower colour. Accumulation of cyanidin was observed in the tepals of 'Perth' and 'Gran Tourismo'. High amounts of dihydrokaempferol accumulated in tepals of all four lily cultivars confirming the expression and functionality of *CHSa, CHSb, CHIa, CHIb* and *F3H* in the pathway. The elevated expression of the structural genes is strongly correlated with the expression of *MYB12* and *MYB15*.

Keywords: *Lilium longiflorum,* Oriental lilies, anthocyanin, gene expression, flower development, colour.

Introduction

The genus *Lilium* (Liliaceae) consists of more than 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), 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* nowadays has become more famous among growers and consumers because of several positive traits such as beautiful flowers, early flowering, easy to grow and propagate, and vase life quality (Martens et al., 2003). Since this species only exist in purely white-flowered cultivars, it is believe that varying its flower colour would be of a great potential commercial value. However, modification of flower colour in this species by conventional breeding could not be done yet due to the sterility of hybrids produced by intersectional crosses with coloured lily section representatives (Karlov et al., 1999). 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 (Nørbæk and Kondo, 1999). The biosynthetic pathway of anthocyanins has been extensively studied and is conserved among many plant species (Winkel-Shirley, 2001). 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 (Nakatsuka et al., 2003; Liu et al., 2011; Lai et al., 2012; Suzuki et al., 2015). The transcription of anthocyanin biosynthetic genes in lilies is assumed to be regulated by an interaction between *MYB12* and *bHLH2* transcription factors (Nakatsuka et al., 2009; Yamagishi, 2011; Yamagishi et al., 2012). Recently, *MYB15* from *Lilium regale* was discovered, controlling anthocyanin pigmentation in the flower buds, leaves and bracts (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 of *LhMYB12* was determined and found to be insufficient (Yamagishi et al., 2012). In the white-flowered Oriental hybrid lily cultivar 'Casa Blanca' and its putative parent *Lilium auratum* var. *platyphyllum,* the *MYB12* 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 (Yamagishi, 2011; Yamagishi et al., 2012). The wild species *Lilium speciosum* having white tepals and red anthers contained a *LsMYB12* gene that was identical to *LhMYB12* of 'Rialto'. This occurrence was considered to be the cause of white tepals in *L. speciosum* white/red line. However, in the white/yellow line (i.e. white tepals/yellow anthers), a nonsense mutation in *DFR* 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 of various genes 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 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. This information can be used in the future to generate new *L. longiflorum* or Oriental lily hybrid cultivars with novel flower colours.

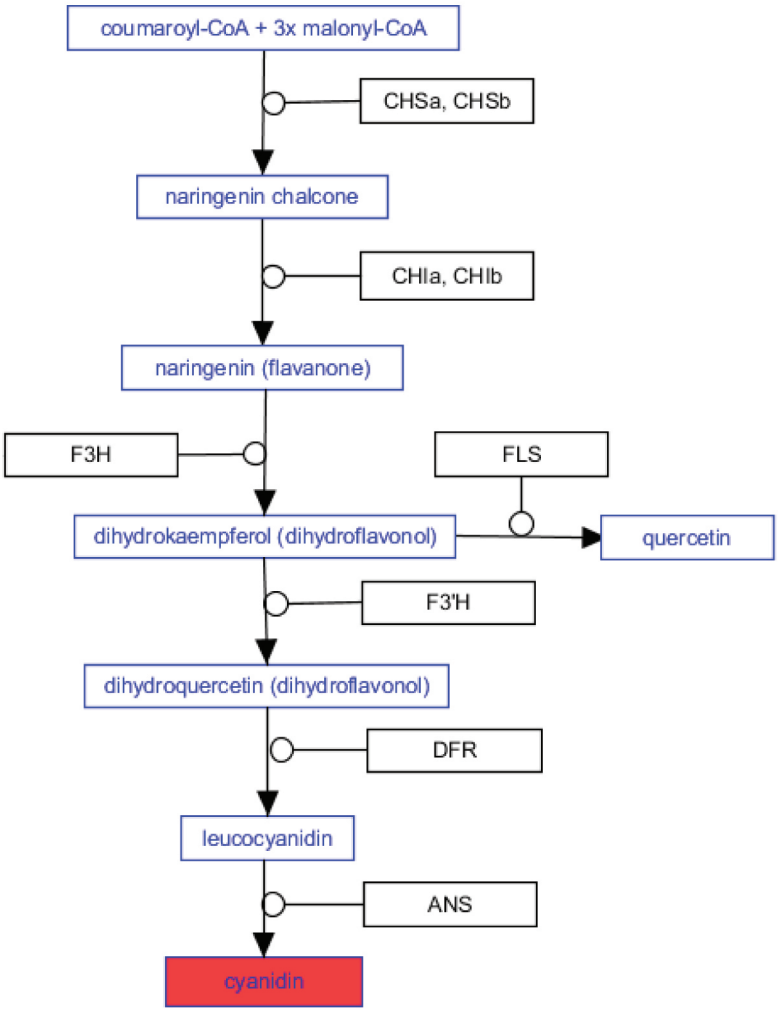


Fig. 1. Simplified anthocyanin biosynthetic pathway in Oriental and Asiatic lilies. *CHSa, chalcone synthase a; CHSb, chalcone synthase b; CHIa, chalcone isomerase a; CHIb, chalcone isomerase b; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol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

Materials and Methods

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 x 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 (more than 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.

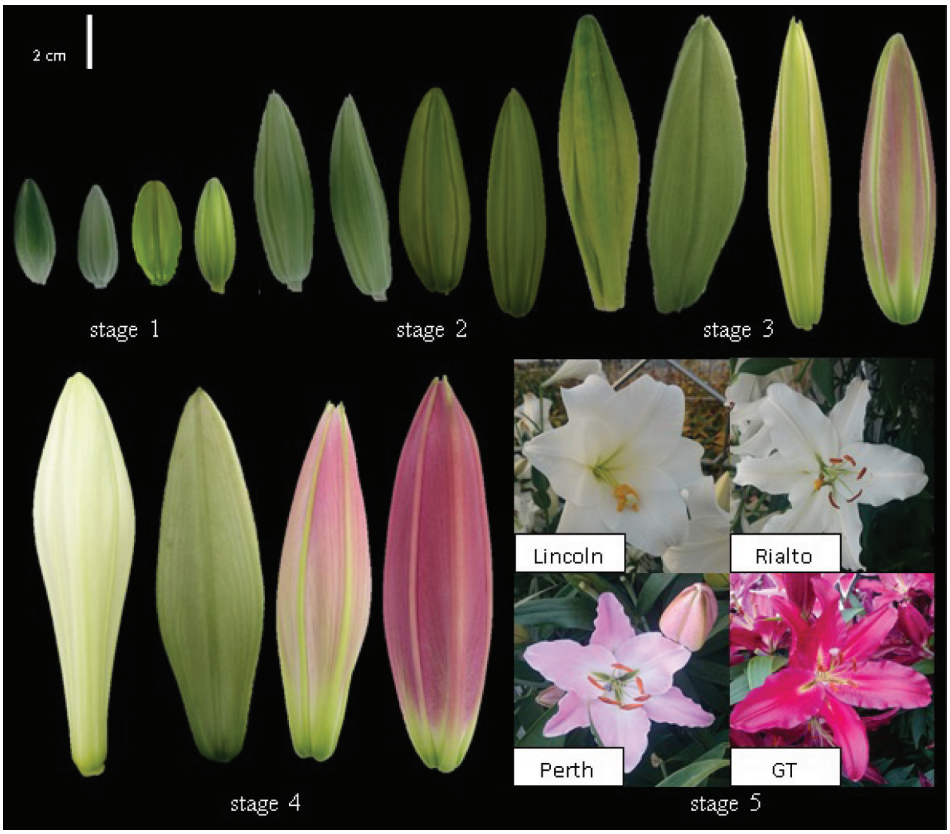


Fig. 2. Five flower developmental stages in *Lilium* cultivars used in this study. Flower buds from left: 'Lincoln'; 'Rialto'; 'Perth' and GT, 'Gran Tourismo'.

PCR 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 instruction. The final PCR reaction mixture (20 µL) contained 2 µL of each sample's gDNA as a template, 2 µL of 2.5 mM 10X 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 milliQ water. *CHSb, CHIa* and *ANS* were amplified using primers from Suzuki et al. (2015). To amplify *F3H, DFR, MYB12, MYB15* and *bHLH2*, gene specific primers were designed based on multiple alignment 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). Subsequently, the amino acid sequences were used for 3D secondary structure predictions in Phyre^2 (http://www.sbg.ic.ac.uk/~phyre2) and PDB Viewer was used for 3D structure alignments.

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 (BHA) in methanol. Three replicates of each cultivar in each stage were used for extraction. The extraction mixture was sonicated for 15 min in an ultrasonic cleaner (VWR International, 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 2N HCl was heated in a heating block set for 120 min at 99ºC.

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). Five concentrations (0.01, 0.025, 0.05, 0.1 and 0.2 mg/mL) of cyanidin chloride, dihydrokaempferol, dihydroquercetin and quercetin were used to make calibration plots with correlation coefficients of 0.99998, 0.99843 and 0.99991, respectively. Quantification of anthocyanins and related compounds was performed by correlating the chromatographic peak area with concentrations in accordance with the calibration plot of the corresponding external standard.

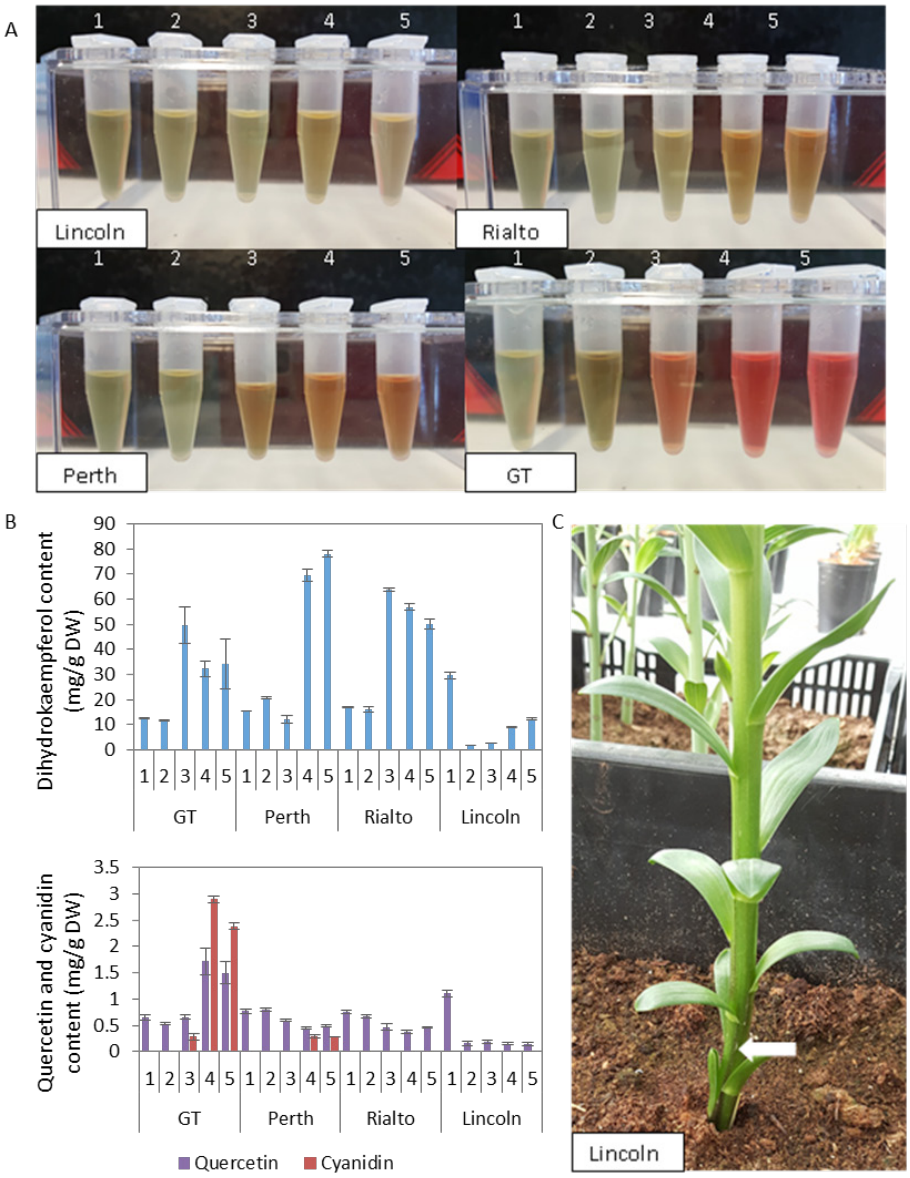


Fig. 3 (A) Acid hydrolysed extracts used for HPLC analysis. (B) Anthocyanin contents in tepals of four *Lilium* cultivars during five flower developmental stages. Vertical bars indicate the standard error of three technical replicates. (C) 'Lincoln' plant showing a streak of anthocyanin pigmentation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

RNA isolation and cDNA synthesis

Total RNA was isolated from tepals (100 mg fresh weight) using the 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 10X DnaseI reaction buffer, 1 µL DnaseI and milliQ water adjusted to a total volume of 10 µL was incubated at 20˚C for 15 min. 1 µL 25mM 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, total reaction mixture (20 µL) consisting of 11 µL Dnase treated RNA, 4 µL 5X 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 milliQ water and kept at 4˚C until further use.

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 2X iQ SYBR GREEN super mix 2X 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 milliQ water. Cycling conditions were: preheating at 95˚C for 3 min, 40 cycles of 95˚C for 15 sec 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 sec. 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, the 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.

Statistical data analysis

Data was subjected to one-way analysis of variance (ANOVA) and means were compared using Duncan's multiple range test at $P<0.05$. Spearman correlation coefficient between gene expressions involved in anthocyanin formation in the Oriental lily cultivars 'Gran Tourismo' and 'Perth' was calculated using GenStat 18th edition.

Construction of *MYB12* expression vectors

MYB12 gene was amplified by PCR from cDNA of Oriental lily hybrid cultivars 'Gran Tourismo', 'Perth' and 'Rialto'. The primers were extended with restriction sites to accommodate the directional cloning as DNA insert (Table S3). PCR products of each NcoI-*MYB12*-BglII were cloned into intermediate vector using Zero Blunt TOPO cloning kit (Invitrogen) and sequenced. The *MYB12* coding sequences were digested from TOPO vector using restriction enzymes. All the digestions were performed at 37˚C in water bath for 1 hour. Complete digestion products were separated on agarose gel and the fragments were purified from gel. Gel purification was performed using Zymoclean gel extraction kit following the protocol given by the manufacturer. The digested fragments were introduced into restriction sites in the pRAPAM vector via NcoI/BglII digestion. In the resulting plasmids, the gene was under the control of the constitutive cauliflower mosaic virus 35S promoter and NOS terminator. The plasmids were further digested with AscI/PacI, and the fragment obtained was inserted into AscI/PacI site in the expression vectors pBinPLUS (van Engelen et al., 1995) containing kanamycin selection to create pBinPLUS:*LhGtMYB12,* pBinPLUS:*LhPerMYB12* and pBinPLUS:*LhRiaMYB12* plasmids. Finally, all the resultant plasmids were introduced into *A. tumefaciens* strain AGL0 (Lazo et al., 1991) by electroporation (Wirth et al., 1989).

Transient transformation in 'Perth' tepals

A single colony of *Agrobacterium* was inoculated in 10 mL liquid Luria-Bertani broth (LB) medium containing 50 mg/L kanamycin and 50 mg/L rifampicin and was grown overnight at 28°C in a shaker (150 rpm) to an OD_{600} ± 1.0. In order to keep the culture in exponential phase of growth, it was diluted 1:10 after 24 hours. The following day, *Agrobacterium* was pelleted by centrifugation at 3000 rpm for 15 mins, the supernatant was discarded, and the pellet was washed once with sterile demineralized water. Subsequently, the *Agrobacterium* suspension was centrifuged, the supernatant was discarded, and the pellet was resuspended in MMA medium (20 g/L sucrose, 5 g/L MS salts (no vitamins), 1.95 g/L MES, 100 µM acetosyringone, pH was adjusted to 5.6 with NaOH, filter-sterilized) at a bacterial density of OD_{600} 1.0 (Yasmin and Debener, 2010; Pinthong et al., 2014). Paclobutrazol (PBZ) at a final concentration of 10 µM was added to the infiltration buffer to enhance transformation efficiency (Hasan Nudin et al., 2015). *Agrobacterium* suspensions were then incubated at room temperature for 1-3 hours for acclimatization before use. *Agrobacterium* containing

pCAMBIA+GUS (Cambia, Australia) was used as a positive control in this experiment. The middle of outer tepals of an intact and closed flower bud (i.e. before anthesis) were pricked by a sterile syringe-needle to facilitate penetration, and subsequently infiltrated with 0.5 mL of *Agrobacterium* suspension using a 1 mL needleless syringe. For each construct, three flowers from six plants which made a total of 18 flowers were infiltrated. The infiltrated plants were kept in a greenhouse with a 16/8 h light-dark photoperiod, temperature at 21˚C and relative humidity at 60%. At 6 days after the infiltration, about 3 cm x 3 cm of infiltrated area in tepals were harvested for anthocyanin measurement and qPCR analysis. The samples were pooled for each treatment, immediately frozen in liquid nitrogen and kept in a -80˚C freezer until use. The relative changes in gene expression were determined based on the $2^{-\delta\delta Ct}$ calculation method, where δCt = Ct (target gene) – Ct (*GAPDH*), and $\delta\delta Ct = \delta Ct$ (treated) – δCt (control) (Livak and Schmittgen, 2001). Three biological replicates of each cultivar in each stage were used in the analysis.

Results

Presence of genes involved in the anthocyanin pathway

The presence 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 was confirmed by sequencing the PCR products and their deduced amino acid sequences were checked by Blastp search (Table S4). Amplified fragments suggested that all three Oriental lily cultivars 'Rialto', 'Perth' and 'Gran Tourismo' contained all eight structural genes (*CHSa, CHSb, CHIa, CHIb, F3H, F3'H, DFR, ANS*) and three transcription factors (*MYB12, MYB15, bHLH2*), while for *L. longiflorum* cultivar 'Lincoln' only the presence of six structural genes (*CHSa, CHSb, CHIa, CHIb, F3H, ANS*) and two transcription factors (*MYB12, MYB15*) could be confirmed in this way. We failed to amplify *F3'H, DFR* and *bHLH2* from '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 be responsible for the absence of anthocyanin accumulation in the white tepals of 'Lincoln'. On the other hand, the *DFR* gene was found to be present in 'Rialto', however expression of this gene could not be demonstrated.

Sequences of *DFR, ANS, MYB12*

Given the presence of *DFR* but the absence of expression in 'Rialto' as mentioned above, we hypothesized that there is a defect in this gene that can impair its function in addition to a mutation in the *MYB12* transcription factor that weakened the expression of anthocyanin structural genes (*DFR* and *ANS*). To test these hypotheses, full-length or partial amino acid sequences of *DFR, ANS* and *MYB12* from *Lilium* cultivars were deduced and analysed.

A full-length *DFR* sequence from 'Gran Tourismo' and 'Perth' together with a partial sequence from 'Rialto' were compared with sequences from *L. speciosum* (Fig. 4). No sequence of *DFR* from 'Lincoln' was determined, since no amplified fragment could be obtained from cDNA and gDNA templates. *DFR*-rr1 from *L. speciosum* having red tepals and red anthers differed from *DFR* sequences from 'Gran Tourismo' and 'Perth' by three unique amino acid changes (V to G, M to V and M to I). 'Gran Tourismo' *DFR* sequence was similar to that of 'Perth', except for two amino acid changes from isoleucine (I) to threonine (T) and glutamine (Q) to glutamic acid (E). The 'Rialto' *DFR* sequence resembled *DFR*-wr from *L. speciosum* having white tepals and red anthers, which was also identical to *DFR*-rr2 from *L. speciosum* having red tepals and red anthers. However, 'Rialto' *DFR* 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 *DFR* partial sequence from 'Rialto'.

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). 'Gran Tourismo' *ANS* differed from the ones in 'Perth' and 'Rialto' only by two amino acid changes from valine (V) to glycine (G) and glutamine (Q) to histidine (H). However, the sequence of *ANS* in 'Lincoln' was not determined at the positions where the two changes have been detected.

A full-length sequence of *MYB12* from 'Gran Tourismo', 'Perth' and 'Rialto' and a partial sequence of *MYB12* from 'Lincoln' were identified and compared (Fig. 6). The *MYB12* sequence from 'Lincoln' was outside of the R2 and R3 repeats, toward the C-terminal, and contained five unique amino acid changes. The 'Gran Tourismo' *MYB12* contained eight unique changes compared to that of 'Perth' and 'Rialto'. However, 'Perth' *MYB12* sequence was identical to 'Rialto', and both contained leucine (L) instead of tryptophan (W) in one of the highly conserved W residues in the R2 repeat.

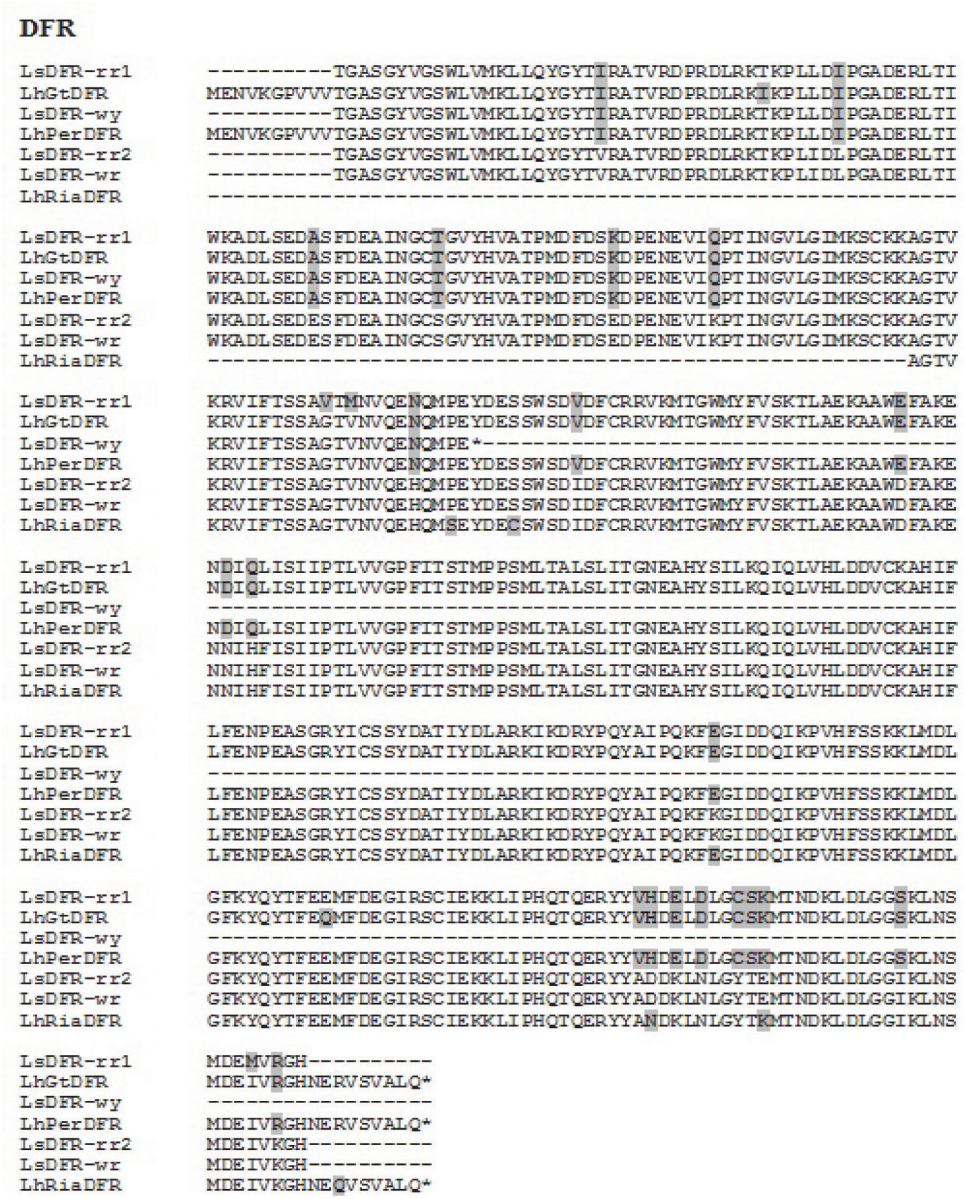


Fig. 4. Multiple sequence alignments of *Lilium* DFR. Letters on grey background indicate amino acid substitutions. Stop codon is marked with an asterisk (*). Lh, *L. hybrida*; Ll, *L. longiflorum*; Gt, 'Gran Tourismo'; Per, 'Perth'; Ria, 'Rialto'; Lin, 'Lincoln'; Ls, *L. speciosum*; rr, red/red line, wy, white/yellow line, wr, white/red line of *L. speciosum*.

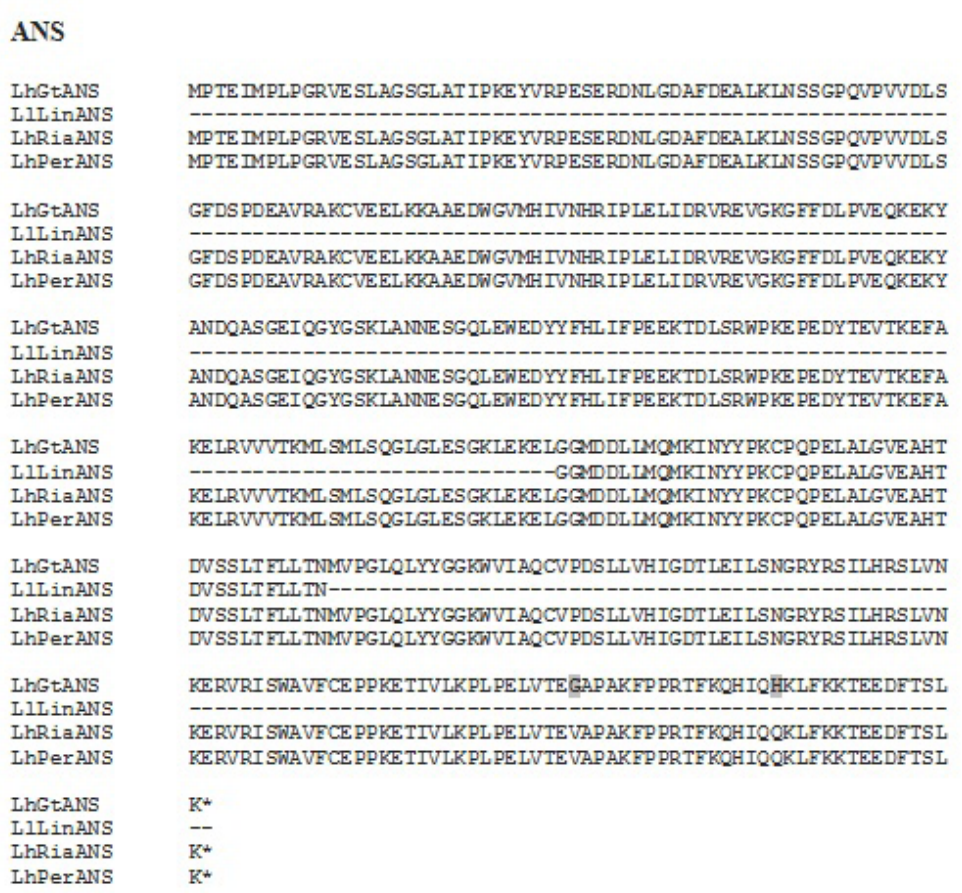


Fig. 5. Multiple sequence alignments of *Lilium* ANS. Letters on grey background indicate amino acid substitutions. Stop codon is marked with an asterisk (*). Lh, *L. hybrida*; Ll, *L. longiflorum*; Gt, 'Gran Tourismo'; Per, 'Perth'; Ria, 'Rialto'; Lin, 'Lincoln'.

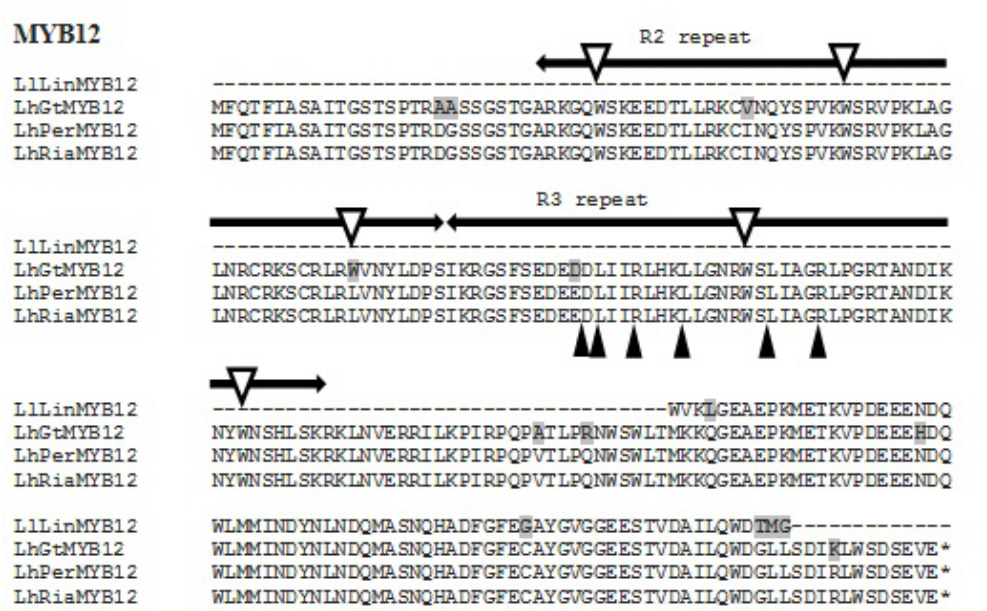


Fig. 6. Multiple sequence alignments of *Lilium* MYB12. Letters on grey background indicate amino acid substitutions. Stop codon is marked with an asterisk (*). White arrowheads indicate the conserved W residues in the R2 and R3 repeats. Black arrowheads indicate the motif [D/E]Lx2[K/R]x3Lx6Lx3R in the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

Anthocyanin accumulation in tepals

Accumulation of cyanidin (anthocyanidin), 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 buds, peaked at stage 4 buds, and then slightly dropped at stage 5 open flowers. 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 that of the three other Oriental lily cultivars, the overall results gave an indication for the functionality in all cultivars of the early structural genes (*CHSa, CHSb, CHIa, CHIb, F3H*) in the pathway and of the transcription factors (*MYB12* and *MYB15*), which are thought to

activate the transcription of 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 *F3'H* and *FLS* activities. However, later the rise of dihydrokaempferol, for example at stage 5, did not lead to 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 *FLS* must be rather high in those three Oriental cultivars compared to 'Lincoln'. Dihydroquercetin was not detected in all four cultivars. However, in 'Perth' and 'Gran Tourismo' where anthocyanin was detected, this must mean that presumably *DFR* was very active in the later stages converting any dihydroquercetin into leucocyanidin at the moment it was made.

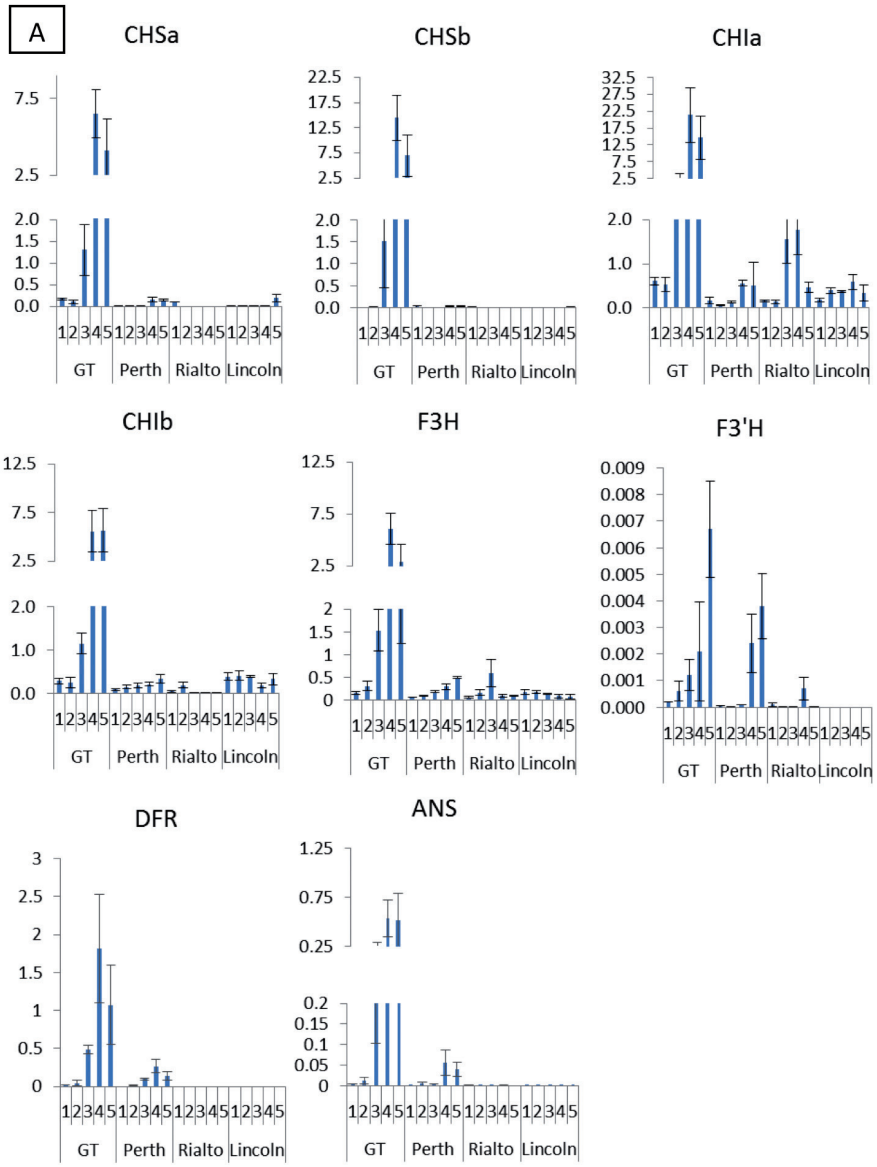
Expression of anthocyanin biosynthesis-related genes

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 expression level was categorized into four categories; very faint (0<RGE<0.1), low (0.1<RGE<0.5), moderate (0.5<RGE<2) and high (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 buds. In 'Perth', those early structural genes were lowly expressed, except for *CHSb* which fell in category 'very faint'. 'Rialto' had moderately expressed *CHIa* and *F3H,* lowly expressed *CHIb*, but no or very faint expression of *CHSa* and *CHSb*. In 'Lincoln', those early structural genes were lowly expressed, but no or very faint expression of *CHSb* was observed. The expression of *F3'H* gene was generally very faint in all four cultivars, in which 'Gran Tourismo' stage 5 open flowers had the highest expression level of *F3'H* followed by 'Perth' stage 5 open flower. Nevertheless, no expression of *F3'H* was detected in 'Lincoln'.

The structural gene *DFR* was moderately expressed in 'Gran Tourismo' but lowly expressed in 'Perth'. 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 *DFR* was observed in 'Rialto' and 'Lincoln'. Also, *ANS* was very faintly expressed in 'Rialto', 'Lincoln' and 'Perth'. In 'Gran Tourismo', *ANS* was lowly expressed.

The expression levels of transcription factors (*MYB12, MYB15* and *bHLH2*) were as follows. In 'Gran Tourismo', *MYB12* was very faintly expressed in stages 1 and 2 buds, upregulated at stage 3 buds, peaked at stage 4 buds, and then dropped at stage 5 open flowers. A similar pattern was observed in 'Perth' and 'Rialto', but the levels were very much lower than that of 'Gran Tourismo'. The white-flowered cultivar 'Lincoln' had very faintly expressed *MYB12* in tepals. Another *MYB* transcription factor, *MYB15* was very

faintly expressed in all four cultivars with ‘Gran Tourismo’ showing the highest expression level. The ‘Gran Tourismo’ *MYB12* and *MYB15* showed an expression pattern similar to that of *CHSa, CHSb, CHIa, F3H, DFR* and *ANS* transcription profiles. *MYB15* was detected in the pink cultivar ‘Perth’ and its expression pattern was parallel to that of *DFR* and *ANS*. In contrast, a different expression pattern was observed in *bHLH2*. The transcription of *bHLH2* decreased over the flowering stages in ‘Perth’ and ‘Rialto’. In accordance with the absence of the *bHLH2* gene gDNA sequence in ‘Lincoln’, no expression of the gene could be seen. Unexpectedly, *bHLH2* was only very faintly expressed in ‘Gran Tourismo’.



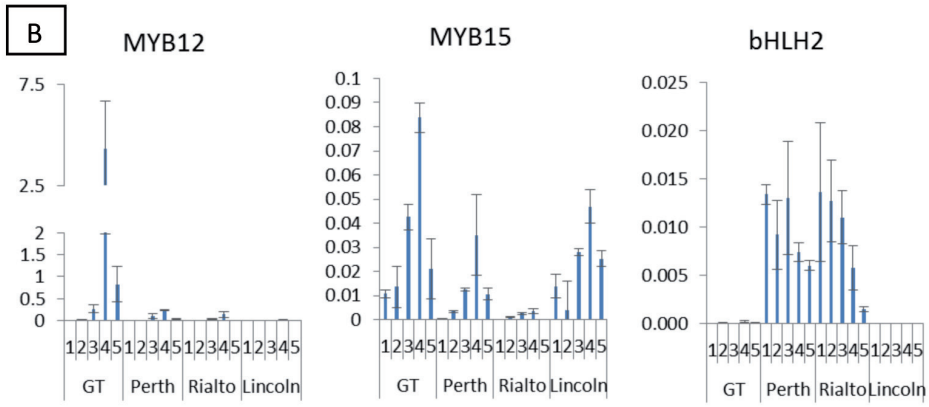


Fig. 7. Relative levels of gene expression of (A) eight structural genes and (B) three transcription factors during five flower developmental stages in Oriental hybrid lily cultivars 'Gran Tourismo', 'Perth', 'Rialto' and *L. longiflorum* cultivar 'Lincoln'. Vertical bars indicate the standard error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

Regulation patterns of transcription factors

The gene expression data showed that many structural genes display similar expression patterns as *MYB12* and *MYB15*, suggesting some co-regulation. However, *bHLH2* 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 the coloured cultivars 'Gran Tourismo' and 'Perth' was performed (Fig. 8). The expression of all structural genes was strongly correlated with each other (all rho above 0.90), with the exception of *F3'H*, which has weaker correlation coefficient (rho=0.34 to 0.56). The transcription factor, *MYB12* showed a positive correlation with all the structural genes (rho=0.72 to 0.90), including *F3'H* (rho=0.32). *MYB15* was also positively correlated with all the structural genes although the correlation coefficient was generally weaker than that of *MYB12*. However, all the structural genes and the *MYBs* were negatively correlated with *bHLH2*. The correlation analysis suggested that both *MYB12* and *MYB15* play an important role in regulating the anthocyanin structural genes in tepals of 'Gran Tourismo' and 'Perth'.

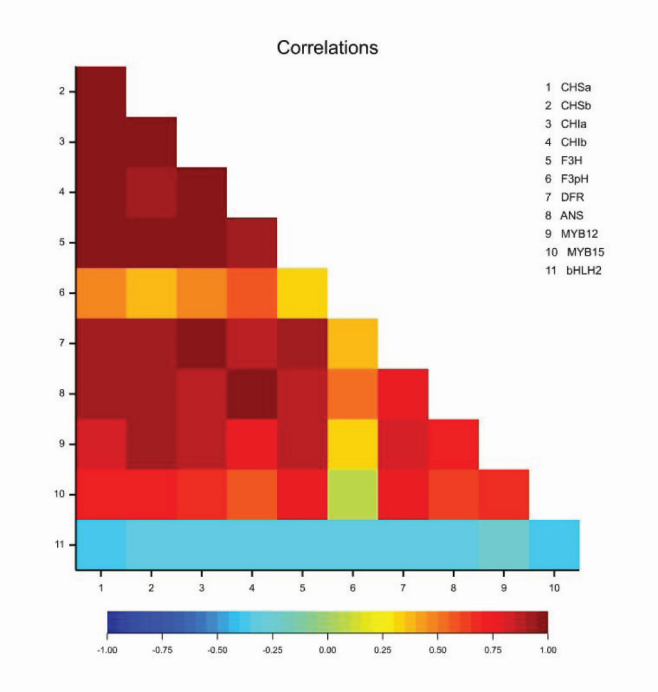


Fig. 8. Correlations between gene expressions involved in colouration in lily cultivars 'Gran Tourismo' and 'Perth'. Colour intensity represents Spearman rank correlation coefficient (*rho*) value. F3pH means *F3'H*.

***MYB12* induced anthocyanin accumulation in 'Perth' tepals**

Changes in anthocyanin content (Fig. 9) and gene expression (Fig. 10) in 'Perth' tepals that have been infiltrated with *MYB12* from 'Gran Tourismo', 'Perth' itself, and 'Rialto' were recorded. Among the three cultivars, introduction of 'Gran Tourismo' *MYB12* showed the highest increase of expression of *MYB12* (5-fold) measured in 'Perth' tepals, followed by the one introduced by 'Rialto' *MYB12* (1-fold), and then by 'Perth' *MYB12* itself (0.5-fold) which was slightly higher but not significantly different from *GUS* control (0.4-fold). The expression pattern of *MYB12* was parallel with *DFR* and *ANS* expression patterns, in which the introduction of 'Gran Tourismo' *MYB12* led to the highest increase of expression of endogenous *DFR* to approximately 33-fold and of *ANS* to about 20-fold, resulting in a deeper pink colouration and a higher cyanidin content in 'Perth' tepals measured at 0.61 mg/g. As expected, 'Rialto' and 'Perth' *MYB12* showed less stimulating effects on the expression of *DFR* (12-fold) and cyanidin content (0.55 mg/g) as compared to 'Gran Tourismo' *MYB12*. As a control, introduction of *GUS* plasmid seemed to have little effect in anthocyanin accumulation, where the expression of *F3'H, ANS* and *MYB12* were slightly upregulated, but *DFR* was not.

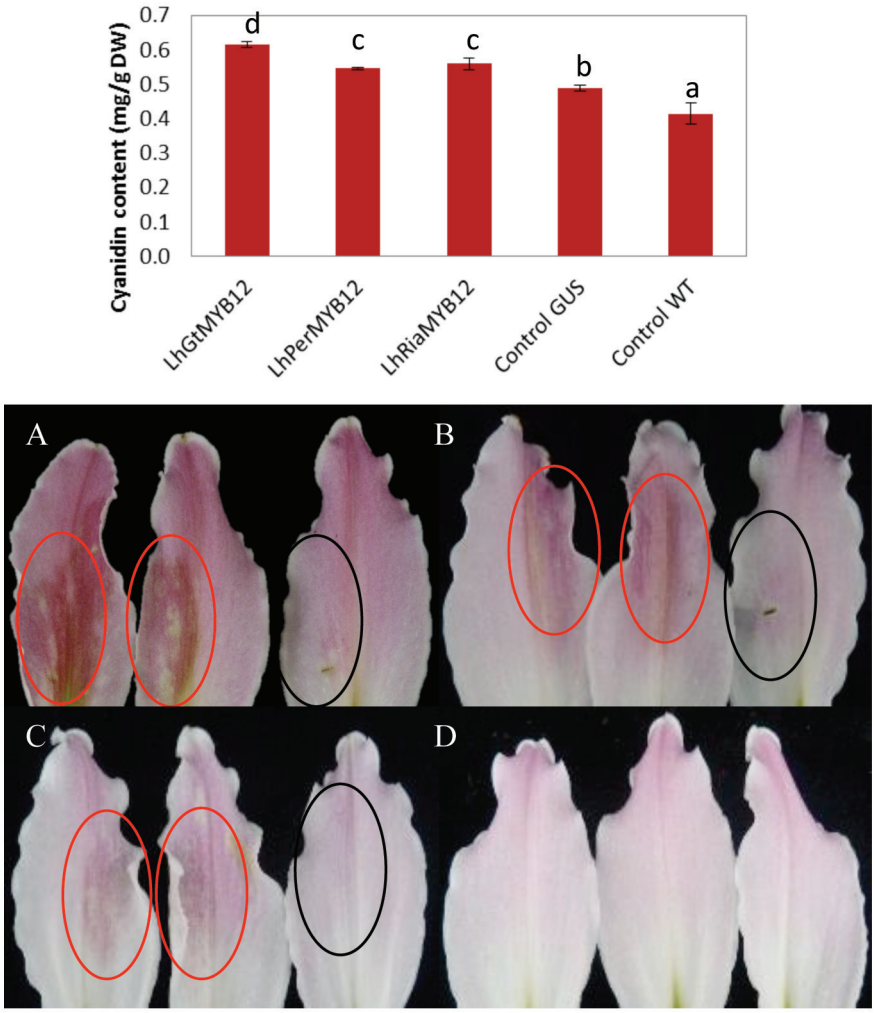


Fig. 9. Cyanidin accumulation (mg/g dry weight) showing deeper pink colouration at the infiltrated area of 'Perth' outer tepals. Vertical bars indicate the standard error of three technical replicates. In each flower, two outer tepals were infiltrated with (A) *LhGtMYB12,* (B) *LhPerMYB12,* (C) *LhRiaMYB12* (shown in red circles) and one outer tepal was infiltrated with *GUS* as a positive control of *Agrobacterium* (shown in black circles). (D) Control wild type (WT) tepals.

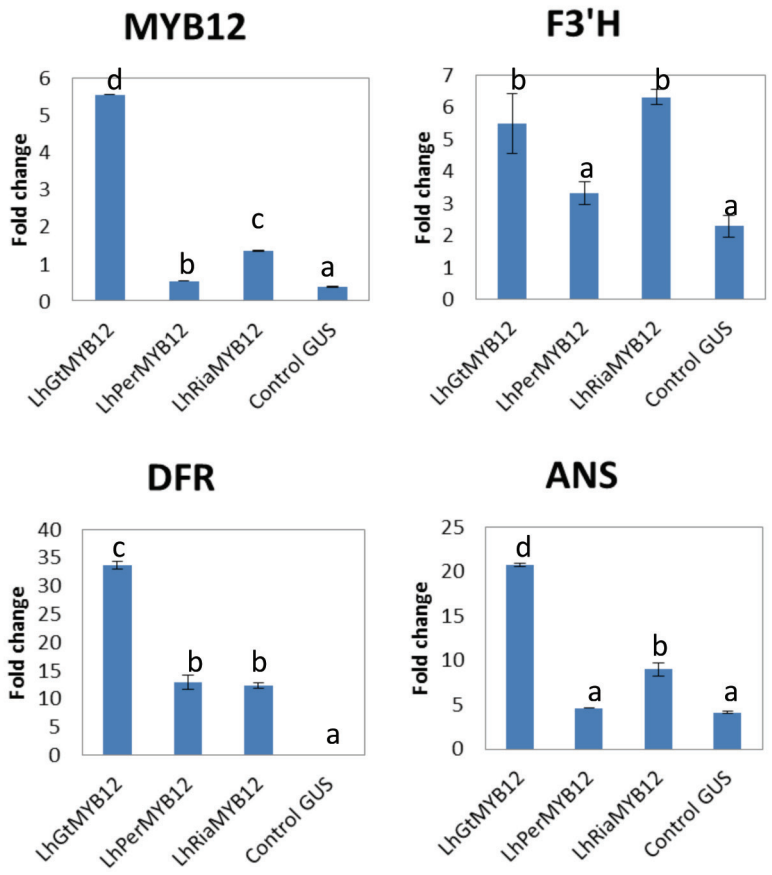


Fig. 10. Relative gene expression patterns in 'Perth' tepals infiltrated with *LhGtMYB12, LhPerMYB12, LhRiaMYB12,* and *GUS* as a positive control. Error bars indicate the standard error of the mean ± SE of three replicate measurements. Lh, *L. hybrida*; Gt, 'Gran Tourismo'; Per, 'Perth'; Ria, 'Rialto'; Lin, 'Lincoln'. Vertical bars indicate the standard error of three biological replicates.

Discussion

For improvement of anthocyanin engineering strategies, a careful characterisation of the structural genes and transcription factors involved in the 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 (*F3'H* and *DFR*) and one transcription factor (*bHLH2*), possibly responsible for our lack of success in determining expression by measuring transcript levels of the genes by qPCR. In 'Rialto', *DFR* was present, but it was not transcribed. No premature stop codon that can impair *DFR* protein function was found in 'Rialto', as was found in *L. speciosum* having white tepals and yellow anthers (Suzuki et al., 2015). *ANS* was hardly expressed in both white-flowered 'Rialto' and 'Lincoln'. However, *ANS* amino acid sequence in 'Rialto' resembled the sequence in 'Perth', indicating no amino acid mutation in *ANS* 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. Only the sequence of 'Perth' *MYB15* could not be obtained, but the presence of this gene in 'Perth' was proven by qPCR analysis (Fig. 7). The RGE levels varied during flower developmental stages.

In many *Lilium* species, *MYB12* usually interacts co-ordinately with *bHLH2* forming a *MYB12/bHLH2* complex to upregulate the transcription of structural genes (Nakatsuka et al., 2009; Yamagishi, 2011; Yamagishi et al., 2012; Lai et al., 2011). 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 (Yamagishi, 2011; Suzuki et al., 2015). We also found this W-to-L substitution in 'Perth' *MYB12* (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 required to maintain the function of *R2R3-MYB* (Yamagishi, 2011), our data suggested that the function of *MYB12* containing W-to-L amino acid substitution in the R2 repeat from 'Perth' and 'Rialto' tepals seemed to be maintained. Firstly, because 'Perth' has pink tepals. Secondly, because the structural genes *DFR* and *ANS* which are supposed to be regulated by *MYB12* were transcribed in 'Perth' tepals, signifying the functionality of the *MYB12* gene. Thirdly, 'Rialto' has red anthers (see Fig. 2, stage 5), which also suggests the functionality of the anthocyanin biosynthetic pathway, and finally, transient expression assays have determined the function of *MYB12* from 'Gran Tourismo', 'Perth' and 'Rialto' by stimulation of cyanidin content in 'Perth' tepals through upregulation of *F3'H, DFR* and *ANS* (Figs. 9-10). 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 observations that the expression levels of *MYB12* in 'Perth' and 'Rialto' were relatively low compared to the ones in 'Gran Tourismo', and the expression levels of endogenous *DFR* and *ANS* in 'Perth' tepals expressing 'Perth' and 'Rialto' *MYB12* were much lower than those induced by 'Gran Tourismo' *MYB12*.

In 'Gran Tourismo', *MYB12* having the conserved W residues was hardly expressed in stage 1 and 2 buds, upregulated at stage 3 buds, peaked at stage 4 buds, and slightly dropped at stage 5, open flowers. The expression patterns of the newly discovered *MYB15* from *L. regale* (Yamagishi, 2016) and *MYB12* from the Oriental cultivars were similar, although the expression levels were much lower compared to *MYB12*. The expression of *MYB15* was detected in 'Perth', but not or very weakly in 'Rialto', and the expression profile was parallel as that of *DFR* and *ANS*. Anthocyanin structural genes showed a similar expression pattern as the *MYBs* 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 *bHLH2* showed a negative correlation to those of the structural genes in both coloured cultivars and the transcription level of *bHLH2* was extremely low in the red tepals of 'Gran Tourismo'. This result indicated that *bHLH2* alone is unable to induce the expression of structural genes in the tepals but that is primarily due to the control of *MYB12* and *MYB15*, and perhaps only a few transcripts of *bHLH2* are required as co-factor to potentiate the function of the *R2R3-MYB*. It was demonstrated in *N. benthamiana* that introduction and expression of *bHLH2* from *L. regale* did not stimulate the transcription of *DFR* and *ANS* (Yamagishi, 2016). However, expression of *bHLH2* together with *MYB15* enhanced the transcription of *DFR* and *ANS*, and the levels were higher than that induced by *MYB15* alone. Not only in lily, the key role of *R2R3-MYB* transcription factors in inducing the transcription of anthocyanin structural genes has been reported, but also 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 *bHLHs* transcription factors that are not yet characterized in *Lilium* species.

Transcription profiles of eight structural genes (*CHSa, CHSb, CHIa, CHIb, F3H, F3'H, DFR* and *ANS*) and metabolite accumulation were examined during flower development in four cultivars of two *Lilium* species. 'Gran Tourismo' and 'Perth' accumulated a single anthocyanidin, i.e. cyanidin, in tepals. The white tepals of 'Rialto' and 'Lincoln' did not contain anthocyanidin. High transcript accumulations were observed in the red tepals of 'Gran Tourismo', followed by the pink tepals of 'Perth'. The white tepals of 'Rialto' and 'Lincoln' showed the lowest transcript accumulations. During flower development of 'Gran Tourismo', no or low anthocyanin structural genes expression was detected at the early bud stage 1 and early bud stage 2, respectively. The expression increased at stage 3 buds, peaked at stage 4 buds, and slightly dropped at stage 5 open flowers. These expression profiles were correlated with the anthocyanidin accumulation profiles. Expression level of *F3'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 *F3'H* transcripts. The transcription of *F3'H* was even lower in 'Perth' and 'Rialto', and could not be determined in 'Lincoln'. As 'Gran Tourismo' did contain cyanidin, *F3'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 and his coworkers (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 all lily tepals. This result indicated the activity and functionality of early structural genes (*CHSa, CHSb, CHIa, CHIb, F3H*) in the pathway in all cultivars, although the expressions of *CHSa* and *CHSb* seemed to be very weak in 'Rialto' and *CHSb* was very weak in 'Lincoln'.

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'. Moreover, the *DFR* sequences from these two coloured cultivars only differed by two amino acid substitutions. The 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 was much smaller. The small decrease between stages 4 and 5 was also seen in cultivar 'Montreux' (Yamagishi et al., 2010). Suzuki and coworkers (2015) found a premature stop codon in *DFR*-wy, and suggested this mutation caused the white colouration in tepals of *L. speciosum* having white tepals and yellow anthers. In 'Lincoln', however, no *DFR* genomic copy could be detected, no *DFR* enzymatic activity 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, suggested the presence of *DFR* 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 a *DFR* gene should be present in this species but we were unable to amplify it by PCR, which was probably due to a high variation in the gene sequences between *Lilium* spp. These observations suggested that genomic PCR does not offer a conclusive evidence for the presence or absence of a particular gene. Therefore, an enzyme activity assay is recommended for future studies.

In this study, we found a *DFR* genomic copy present in 'Rialto', but the gene was not expressed. Four unique amino acid substitutions were found in *DFR* from 'Rialto' compared to other Oriental lilies and *L. speciosum* (Fig. 4). However, no critical mutation causing e.g. a premature stop codon was found, as reported in *DFR*-wy from *L. speciosum* having white tepals and yellow anthers (Suzuki et al., 2015). To gain insight into the potential effects of the mutation points found in *DFR* sequences, we executed a prediction of protein structure using Phyre2 (http://www.sbg.bio.ic.ac.uk/~phyre2) (Kelly et al., 2015). The predicted 3D protein structure of *DFR* genes showed structural differences between Oriental cultivars (Fig. 11). The active site detected as based on Catalytic Site Atlas program (Furnham et al., 2014) and the protein pocket detected by the fpocket2 program (Schmidtke et al., 2011) were observed in all three *DFR* copies checked from 'Gran Tourismo', 'Perth' and 'Rialto'. These results indicated no major defects that could impair DFR protein function. Yet, it is possible that amino acid substitutions in *DFR* from 'Rialto' decreased the binding affinity to dihydroquercetin. According to Johnson and coworkers (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 in a hydrogen bonding residue could show a dramatic decrease in the binding affinity to take up dihydroquercetin as a substrate. Nevertheless, the gene functionality will be studied in the near future by complementation of dfr mutants in *Arabidopsis*.

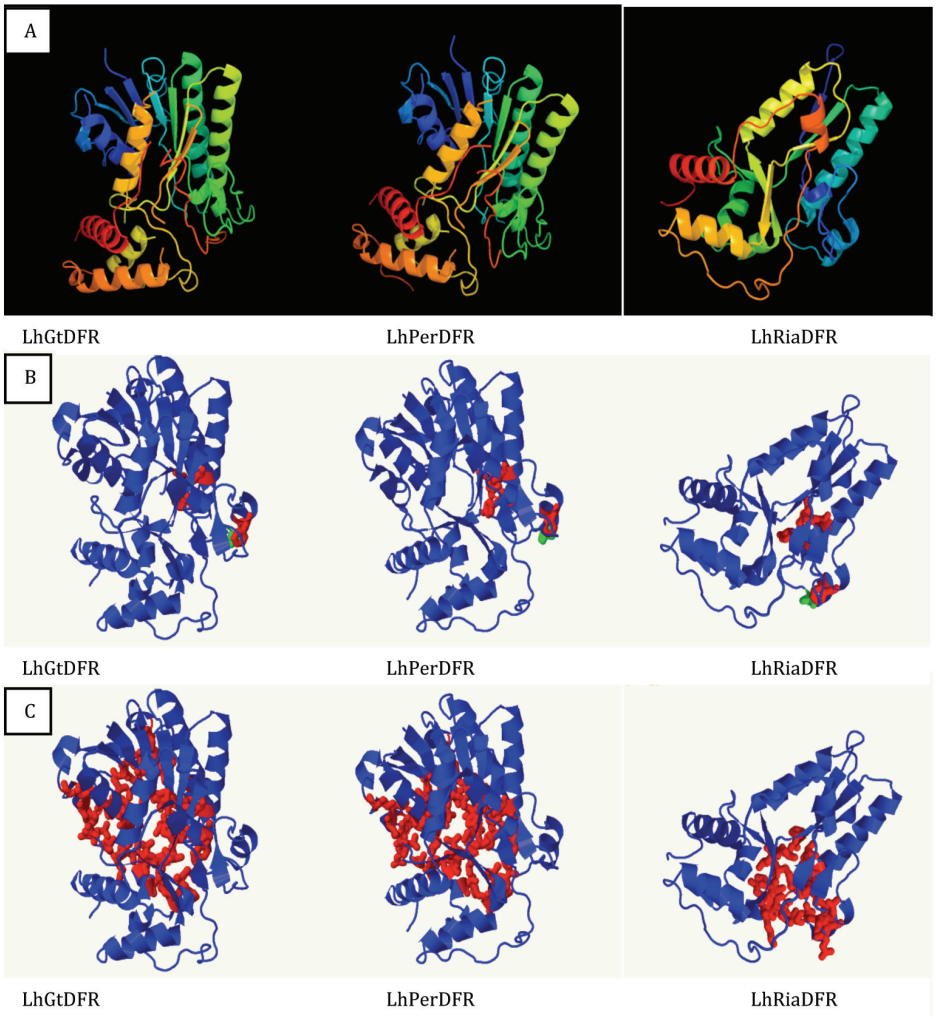


Fig. 11 (A) Prediction of 3D protein structure of *LhGtDFR, LhPerDFR* and *LhRiaDFR* gene. The colouration indicates the N to C direction of the protein starting with red colour at N terminus and reaching blue at C terminus. (B) Active sites are found in the template protein. Red indicates identical residues between query and template and green indicates different residues at this position. (C) Large pockets are frequently found to be the location of active sites. Pockets are shown in wireframe mode, coloured red.

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 of 'Lincoln' and 'Rialto', *ANS* was hardly expressed. Similarly in white tepals of *L. speciosum*, hardly any expression of *ANS* was detected (Suzuki et al., 2015). In addition,

it was demonstrated that lowering *ANS* 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.

In conclusion, anthocyanin accumulation was paralleled by the expression of the structural genes, which in turn were positively correlated with expression of *MYB12* and *MYB15*. On the other hand, *bHLH2* was negatively correlated with the expression of the structural genes and it was known that expression of *bHLH2* alone could not activate the expression of anthocyanin. All the analysed structural genes displayed much higher expression in the red tepals of 'Gran Tourismo' compared to the pink tepals of 'Perth'. High accumulation of dihydrokaempferol in all lilies suggested that the early genes from *CHSa* up to *F3H* were present and functional. Therefore, it was assumed that 'Lincoln' is white most likely due to the absence of two structural 'late' genes (*F3'H* and *DFR*) and one transcription factor gene (*bHLH2*). No transcription of *DFR* seemed the most probable cause of white tepals in 'Rialto'. Our results suggest that developing cyanidin-based flower colour in 'Lincoln' might require an active expression of *bHLH*-type transcription factor together with an *F3'H* gene that is able to catalyse dihydrokaempferol reduction, and expression of *DFR* and *ANS* genes that can efficiently further process reduced dihydroquercetin. 'Rialto' may require the introduction and active expression of *DFR* and *ANS* 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. This study will be followed by another study in which genes will be transiently introduced into lilies tepals and genes expression will be monitored (**Chapter 5**). The findings of this study can pave the path for changing of white-flowered *L. longiflorum* and Oriental lily hybrid lilies into flowers with novel colour.

Table S1. List of primers used for relative gene expression analysis and primer efficiency.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Reference	Primer efficiency
LhGAPDH	CTACTGTGCACGCCATCACT	ACACATCGACAGTGGGAACA	This study	1.9340
LhCHSa	TGGGACTCACCTTCCATCTC	CATGTTTCCGTACTCGCTCA	This study	1.8882
LhCHSb	CTGAAGCTGGCGCTGGACAAAAAG	GGTAGTGATCGGAATGCTGTGAAGA	Yamagishi, 2011	1.8269
LhCHIa	TCCATCCTCTTCACCCAGTC	CCTTGAGAAGCTCGGAAATG	Suzuki et al., 2015	1.8949
LhCHIb	GCGGTCGATAAGTACGAGGA	TCCCACCCAAATACCACTTC	This study	1.9006
LhF3H	TGCCTTTGTTGTCAATCTCG	GCATCCAAAACTGGCTTCTC	This study	1.8993
LsF3'H	ACGCACGACACAAACTTCAG	CGAGTGCTTTCTGGGAAAAG	Suzuki et al., 2015	1.8451
LhDFR	ATATGCCATCCCCCAAAAGT	GCAACCCAAATCCAGTTCAT	Yamagishi, 2011	1.8862
LhANS	GGGGGAATGGATGACCTACT	GTTGGTGAGGAGGAAGGTGA	Suzuki et al., 2015	1.8407
LhMYB12	GGGTGAAGCTGAACCAAAAA	GTCCCATTGGAGAATTGCAT	This study	1.9179
LrMYB15	CTCTTGGGAAACAGGTGGTC	TCGACATGGCCGGTTTTCTG	This study	1.9031
LhbHLH2	GGCCAAGCAACTCAAAAGAG	CAACTCGATATGGGCACCTT	This study	1.8995

Lh or Ls indicates the primers were designed based on the *L. hybrid* or *L. speciosum* sequences, respectively.

Table S2. List of primers used for PCR and sequencing.

Name of primer	Forward primer 5'-3'	Reverse primer 5'-3'	Reference
LsCHSb_1178	CAGTCCAACCATGTCGAAGA	TCGGAAGGCTGTGAAGAACT	Suzuki et al., 2015
LsCHIa_683	AGCTCCGAAGCTGGAGGTC	CACTAACAATGGTAGGCTCTTCCT	Suzuki et al., 2015
LhF3H_940	CGACTACCTTCCTCCCAACA	CAACTTCGGTGGGTTTCTTC	This study
LhGtDFR	ATGGAGAATGTGAAAGGACCCGTGG	TTACTGAAGAGCAACGGAGACTCGT	This study
LsDFR_447	CCAGCCCACAATAAATGGAG	CCACTCGCTTCTGGATTCTC	This study
LhDFR_628	CATGGGATTTTGCAAAGGAG	TGGTCCTCCAAAGCTTACTGA	This study
LhGtANS	ATGCCGACCGAGATCATGCCGTTGC	TCACTTGGGAGAAGTGAAGTCCTCC	This study
LsANS_1062	ACCGAGATCATGCCGTTG	GTCCTCCTCTGTCTTCTTGA	Suzuki et al., 2015
LhF3'H4	TTCACAGCAGGGACAGACAC	CATTGATGGTGCAAGATTCG	This study
LhGtMYB12	ATGTTTCAAACGTTTATTGCCTCCG	TTATTCAACTTCGGAATCACTCCAA	This study
LsMYB12_full	TTTCAAACGTTTATTGCCTCCGC	CAACTTCGGGAATCACTCCAAAG	This study
LhMYB12_full2	GCTTCAACGAGGATGAGGAG	TCATTTGGCGGAAAAACTCT	This study
LhMYB15_600	CATCCCAGTTAGAATGCAGAAA	AAATTATACCAACGTTTGACTGTTCC	This study
LsbHLH2_600	CACTCAACCTCCAACCCAGT	TGCTGTTTGCCAAGAATGAG	This study
LhbHLH2_811	AGGCACACTGCAGGAAGAAC	TACCATCATCACCGACGAGA	This study

Lh or Ls indicates the primers were designed based on the *L. hybrid* or *L. speciosum* sequences, respectively.

Table S3. List of primers used for cloning.

Primer names	Sequences from 5'-3'
GtMYB12_F_NcoI	GTCACCATGGATGTTTCAAACGTTTATTGCCTCCG
GtMYB12_R_BglII	GTCAAGATCTTTATTCAACTTCGGAATCACTCCAA
CaMV35S_F	CTATCCTTCGCAAGACCCTTC
Tnos_R	TGCCAAATGTTTGAACGATC
M13_F	GTAAAACGACGGCCAG
M13_R	CAGGAAACAGCTATGAC

The restriction sites are underlined: NcoI: CCATGG, BglII: AGATCT. Gt indicate the primers were designed based on the Oriental hybrid lily cultivar 'Gran Tourismo'.

Table S4. Blastp top hit of the deduced amino acid sequences

Gene	Nucleotide length (bp)	Deduced amino acids	Blastp top hit	Identity (%)	Accession number
LhGtCHSa	230	74	chalcone synthase [*Lilium speciosum*]	96	BAS69307.1
LhPerCHSa	229	75	chalcone synthase [*Lilium speciosum*]	99	BAS69306.1
LhRiaCHSa	230	76	chalcone synthase [*Lilium speciosum*]	99	BAS69306.1
LlLinCHSa	230	76	chalcone synthase [*Lilium speciosum*]	100	BAS69306.1
LhGtCHSb	1097	360	chalcone synthase [*Lilium speciosum*]	99	BAS69313.1
LhPerCHSb	232	76	chalcone synthase [*Lilium* hybrid division VII]	98	AAD49355.1
LhRiaCHSb	1100	360	chalcone synthase [*Lilium speciosum*]	100	BAS69313.1
LlLinCHSb	1157	326	chalcone synthase [*Lilium regale*]	98	BAU29934.1
LhGtCHIa	580	193	chalcone isomerase [*Lilium speciosum*]	100	BAS69315.1
LhPerCHIa	688	228	chalcone isomerase [*Lilium* hybrid cultivar]	99	AII80421.1
LhRiaCHIa	692	229	chalcone isomerase [*Lilium* hybrid cultivar]	97	AII80421.1
LlLinCHIa	665	221	chalcone isomerase [*Lilium* hybrid cultivar]	96	AII80421.1
LhGtCHIb	223	74	chalcone isomerase [*Lilium speciosum*]	100	BAS69337.1
LhPerCHIb	223	74	chalcone isomerase [*Lilium speciosum*]	100	BAS69337.1
LhRiaCHIb	223	74	chalcone isomerase [*Lilium speciosum*]	100	BAS69337.1
LlLinCHIb	223	74	chalcone isomerase [*Lilium speciosum*]	100	BAS69337.1
LhGtF3H	876	291	flavanone 3-hidroxylase [*Lilium speciosum*]	99	BAS69324.1
LhPerF3H	1016	336	flavanone 3-hidroxylase [*Lilium speciosum*]	98	BAS69324.1

Table S4. Continue

Gene	Nucleotide length (bp)	Deduced amino acids	Blastp top hit	Identity (%)	Accession number
LhRiaF3H	970	319	flavanone 3-hidroxylase [*Lilium speciosum*]	99	BAS69324.1
LlLinF3H	191	63	flavanone 3-hydroxylase [*Lilium* hybrid division I]	100	BAM28970.1
LhGtF3'H	251	83	flavonoid 3'-hydroxylase [*Lilium speciosum*]	100	BAS69327.1
LhPerF3'H	251	83	flavonoid 3'-hydroxylase [*Lilium speciosum*]	100	BAS69327.1
LhRiaF3'H	283	94	flavonoid 3'-hydroxylase [*Lilium* hybrid division I]	89	BAM28972.1
LlLinF3'H	ND				
LhGtDFR	1134	378	dihydroflavonol 4-reductase [*Lilium speciosum*]	99	BAE79202.1
LhPerDFR	1134	378	dihydroflavonol 4-reductase [*Lilium speciosum*]	99	BAE79202.1
LhRiaDFR	801	262	dihydroflavonol 4-reductase [*Lilium speciosum*]	98	BAS69330.1
LlLinDFR	ND				
LhGtANS	1086	362	anthocyanidin synthase [*Lilium* hybrid division VII]	100	ALA55544.1
LhPerANS	1086	362	anthocyanidin synthase [*Lilium speciosum*]	100	BAS69334.1
LhRiaANS	1086	362	anthocyanidin synthase [*Lilium speciosum*]	100	BAS69334.1
LlLinANS	126	42	anthocyanidin synthase [*Lilium speciosum*]	100	BAS69335.1
LhGtMYB12	720	240	transcription factor R2R3-MYB [*Lilium* hybrid division VII]	99	BAJ22983.1
LhPerMYB12	720	240	transcription factor R2R3-MYB [*Lilium* hybrid division VII]	99	BAJ22983.1
LhRiaMYB12	720	240	transcription factor R2R3-MYB [*Lilium* hybrid division VII]	99	BAJ22983.1
LlLinMYB12	212	70	transcription factor R2R3-MYB [*Lilium speciosum*]	94	BAS69296.1

Table S4. Continue

Gene	Nucleotide length (bp)	Deduced amino acids	Blastp top hit	Identity (%)	Accession number
LhGtMYB15	203	67	transcription factor R2R3-MYB [Lilium regale]	96	BAU29930.1
LhPerMYB15	ND				
LhRiaMYB15	203	67	transcription factor R2R3-MYB [Lilium regale]	97	BAU29930.1
LlLinMYB15	203	67	transcription factor R2R3-MYB [Lilium regale]	97	BAU29930.1
LhGtbHLH2	542	180	bHLH transcription factor [Lilium speciosum]	93	BAS69304.1
LhPerbHLH2	232	77	bHLH transcription factor [Lilium speciosum]	100	BAS69304.1
LhRiabHLH2	233	77	bHLH transcription factor [Lilium speciosum]	100	BAS69304.1
LlLinbHLH2	ND				

ND means the sequence is not determined.

CHAPTER 5

Modification of anthocyanin production in *Lilium* tepals by transient expression of target genes

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Abstract

The genus *Lilium* has been one of the most important genera of cut flowers worldwide for many decades. The Oriental and Asiatic hybrids are favoured by the consumers because of their large flowers with broad colour variations. Longiflorum lilies, however, only exist in white-flowered cultivars. Thus, varying its flower colour would be of potential commercial value. We have previously identified molecular mechanisms behind white colouration in the *L. longiflorum* cultivar 'Lincoln' and the white Oriental hybrid lily cultivar 'Rialto'. The absence of *F3'H, DFR* and *bHLH2* genes and insufficient expression of the *ANS* gene, were likely responsible for the white-flower colour in 'Lincoln'. In 'Rialto', the *DFR* gene was present but not expressed. In addition, a single W-to-L amino acid mutation was found in *MYB12*, which was assumed to be at the root of white colouration in 'Rialto' flowers. In this study, we determined experimentally the functionality of the *F3'H* gene sequence from *L. speciosum*, and of the *DFR, ANS* and *MYB12* gene sequences from the Oriental hybrid lily cultivar 'Gran Tourismo' by complementation analysis in *Arabidopsis* mutants and/or transient expression in lily cultivar 'Perth' tepals. The genes were transiently co-expressed with or without snapdragon *ROS1* and *DEL* transcription factor genes in 'Rialto' and 'Lincoln' tepals. We demonstrated that *F3'H* and *ANS* are two functional genes that are able to induce anthocyanin biosynthesis in 'Perth' and restore the gene function in *Arabidopsis* mutants, while introduced *DFR* was not expressed at all. *MYB12* from 'Gran Tourismo' induced anthocyanin accumulation in 'Perth' tepals through activation of *DFR* and *ANS*. However, co-expression of multiple genes failed to induce anthocyanin biosynthesis in 'Rialto' and 'Lincoln' due to the complexity of anthocyanin biosynthetic pathway, in which *DFR* seemed to be the main bottleneck. Thus, a functional and suitable *DFR* gene copy remains to be identified and subsequently tested in both cultivars, alone or together with other anthocyanin genes, in order to produce altered flower colour. The information gained in this study should aid in future efforts in designing rational strategies for modification of flower colours in lilies.

Keywords: *Lilium*, *Arabidopsis* mutants, transient expression, anthocyanin, flower colour

Introduction

Lilium (Liliaceae) consisting of more than 100 species (Comber, 1949; Smyth et al., 1989; Lim et al., 2008) is one of the most important cut flowers worldwide. The Oriental and Asiatic hybrid lilies have been commercially important in the ornamental cut flower industry due to their outstanding flower shape, fragrance and large colour variations (Lim and van Tuyl, 2006; Yamagishi and Akagi, 2013). The Oriental hybrids have flowers in white, pink and cream colours, while the Asiatic hybrids vary from white, yellow, orange, pink, red and also bicoloured with or without spots (Yamagishi, 2013). Lilies from the Longiflorum group only exist in white-flowered cultivars. They are known as Easter lily and often used for religious ceremonies (Grassotti and Gimelli, 2011). Although white is also an important commercial flower colour, broadening the Longiflorum flower colour range would be of potential commercial value, since consumer preferences are changing all the time.

Anthocyanins are pigments that provide colours in red, purple and blue to plant organs. The three major groups of anthocyanidins present in higher plants are cyanidin, pelargonidin and delphinidin (Schwinn and Davies, 2004). Lilies produce cyanidin 3-O-β-rutinoside as a major anthocyanin and cyanidin 3-O-β-rutinoside-7-O-β-glucoside as a minor anthocyanin (Nørbæk and Kondo, 1999), however, they cannot produce delphinidin because of the absence of a *flavonoid 3'5'-hydroxylase (F3'5'H)* gene in the pathway (Martens et al., 2003). The anthocyanin biosynthetic pathway is well characterized, and many genes encoding anthocyanin biosynthetic enzymes have been isolated and identified (Lim et al., 2016). Anthocyanin structural genes, like *phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone-3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), dihydroflavonol 4-reductase (DFR)* and *anthocyanidin synthase (ANS)* have been identified and studied from lilies (Nakatsuka et al., 2003; Liu et al., 2011; Lai et al., 2012; Suzuki et al., 2015).

We previously performed gene expression and anthocyanin measurement studies of three Oriental hybrid lily cultivars 'Gran Tourismo' (red tepals), 'Perth' (pink tepals) and 'Rialto' (white tepals), and one *L. longiflorum* cultivar 'Lincoln' (white tepals) during flower colour development. Anthocyanin structural genes were highly expressed in the red 'Gran Tourismo', followed by the pink 'Perth'. The elevated expression of the structural genes was in agreement with the accumulation of cyanidin. The absence of two structural genes *F3'H*, *DFR* and one transcription factor *basic helix-loop-helix 2* (*bHLH2*) gene likely caused the white colour of 'Lincoln' tepals. Together with that, insufficient expression of *ANS* may have contributed to the white colouration in 'Lincoln'. However in 'Rialto', *DFR* was present but not expressed. In anthocyanin biosynthetic pathways, F3'H which converts dihydrokaempferol into dihydroquercetin is the key enzyme of a branch leading to cyanidin production. DFR which converts dihydroflavonol into leucoanthocyanidin, followed by ANS which take up leucoanthocyanidin to produce cyanidin are two important enzymes during

the late steps of the pathway (Nakatsuka et al., 2003). Therefore, molecular characterization of functional *F3'H, DFR* and *ANS* genes in the regulation of anthocyanin is of importance.

Activation of anthocyanin structural genes in lilies is controlled by *MYB12* and *bHLH2* transcription factors (Nakatsuka et al., 2009; Yamagishi, 2011; Yamagishi et al., 2010; Lai et al., 2011). The *MYB*-type transcription factor has been the most extensively studied and was shown to play the most important role in regulating anthocyanin production, as it can bind directly to the target genes (Hichri et al., 2011). In several plant species, the expression of the *MYB* alone is sufficient to stimulate anthocyanin production, as was found e.g. by overexpression of *PAP1* in *Arabidopsis* (Borevitz et al., 2000; Qiu et al., 2014), of *anthocyanin1* (*ANT1*) in tomato (Schreiber et al., 2012), and of *MYB10* in gerbera, apple, strawberry and potato (Elomaa et al., 2003; Kortstee et al., 2011). By contrast, expression of maize *C1* (a *bHLH*-type) failed to stimulate anthocyanin accumulation in tomato without *LC* (a *MYB*-type) transcription factor (Bovy et al., 2002), suggesting that *bHLH*-type transcription factors are often required to support the function of *MYB*-type transcription factor (Butelli et al., 2008; Maligeppagol et al., 2013; Outchkourov et al., 2014). According to Yamagishi (2011) and coworkers (2010), the Oriental hybrid lily cv. 'Rialto' is white due to a W-to-L amino acid substitution in the R2-repeat of *MYB12*. The *MYB12* gene was expressed, but the mutation has likely disrupted its function. An identical mutation was found in *MYB12* from the wild species *Lilium speciosum* having white tepals and red anthers (Suzuki et al., 2015). The occurrence of this mutation was considered to be the cause of white tepals in both species.

To investigate the hypothesized causes for white flower colour in lily, we determined experimentally the functionality of the *F3'H* gene from *L. speciosum* and of the *DFR, ANS and MYB12* genes from 'Gran Tourismo' by complementation analysis in *Arabidopsis* mutants and/or transient expression in Lily cv. 'Perth' tepals. On top of that, the genes were transiently expressed with or without snapdragon *ROS1* and *DEL* transcription factor genes in 'Perth', 'Rialto' and 'Lincoln' tepals. Based on the results, we then discuss whether the genes under investigation can complement the lacking or mutated genes in the white flowered 'Lincoln' and 'Rialto'.

Materials and Methods

Plant materials

Four lily cultivars including *L. longiflorum* cultivar 'Lincoln' (white tepals), Oriental hybrid lily cultivars 'Rialto' (white tepals) and 'Perth' (pink tepals) and 'Gran Tourismo' (red tepals) were used (Fig. 1). The bulbs were obtained from De Jong Lelies Holland BV (Andijk, The Netherlands) and were stored at -1˚C and transferred to 4˚C overnight before planting. The bulbs were planted in crates (30 cm x 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.

Fig. 1. The four *Lilium* cultivars used in this study were the Oriental hybrid lily cultivars (A) 'Gran Tourismo', (B) 'Perth', (C) 'Rialto and (D) *L. longiflorum* cultivar 'Lincoln'.

Arabidopsis mutant tt3-1 (ABRC stock number: CS84) with Landsberg-0 (Ler) ecotype background, tds4-2 (ABRC stock number: CS2105579) with ecotype Columbia-0 (Col-0) background, tt7-3 (ABRC stock number: CS6509) with Columbia-7 (Col-7) ecotype background were used for plant transformation. Seeds were sown in pots (10.5 cm diameter) in the greenhouse. The greenhouse temperature was maintained at around 21˚C during the day and 19˚C during the night (16/8 h photoperiod). The relative humidity was set at 70%. The soil mixture used in this study was composed of 5% of Swedish sphagnum peat, 41% of grinded clay granules, 5% of garden peat, 4% of spore elements, 33% of steamed compost and 12% of PG-Mix 15-10-20. About 5-week-old plants with several mature flowers on the main stems were used for transformation.

RNA isolation and cDNA synthesis

Total RNA was isolated from lily tepals (100 mg fresh weight) using TRIzol reagent (Invitrogen Thermo Fisher Scientific) according to the manufacturer's protocol. RNA was treated with DNaseI (Invitrogen Thermo Fisher Scientific). The reaction mixture containing 80 µL RNA, 9 µL 10X DnaseI reaction buffer and 1 µL DnaseI was incubated at 20˚C

for 15 min. 10 µL 25 mM EDTA was added to the reaction mixture, which was further incubated at 65˚C for 10 min, to stop the reaction. RNA was purified using RNeasy mini kit (Qiagen, Germany) according to the manufacturer's protocol. The quantity and quality of RNA were determined using Nanodrop1000™ and gel electrophoresis. First-strand cDNA was synthesized using Taqman reverse transcription reagent kit (Life Technologies, Applied Biosystems #N8080234) according to the manufacturer's protocol. In short, total reaction mixture (50 µL) consisted of 1 µg RNA, 5 µL 10X Taqman RT buffer, 11 µL $MgCl_2$, 10 µL dNTP mix, 2.5 µL oligo dT, 1 µL RNase inhibitor, 1.25 µL MultiScribe reverse transcriptase and miliQ water was incubated in PCR machine at 25˚C for 10 min (annealing of primer), 48˚C for 30 min (extension), 95˚C for 5 min (deactivation of reverse transcriptase) and hold at 10˚C. The cDNA (20 ng/µL) was kept at 4˚C until further use.

Construction of expression vectors

DFR, ANS and *MYB12* genes were amplified by PCR from cDNA of Oriental lily hybrid cv. 'Gran Tourismo', and primers were extended with restriction sites to accommodate the directional cloning of DNA amplicons (Table S1). PCR products of SacI-*DFR*-BglII, NcoI-*ANS*-BglII and NcoI-*MYB12*-BglII were cloned into opened pCRII intermediate vectors using Zero Blunt TOPO cloning kit (Invitrogen) and sequenced. The *DFR, ANS* and *MYB12* coding sequences were digested from TOPO vector using restriction enzymes. All the digestions were performed at 37˚C in water bath for 1 hour. Complete digested products were separated on agarose gel and the fragments were gel purified. Gel purification was performed using Zymoclean gel extraction kit following the protocol given by the manufacturer (Zymo Research). The digested fragments were introduced into restriction sites in the pRAPAM vector via SacI/BglII digestion for *DFR*, and NcoI/BglII digestion for *ANS* and *MYB12*. In the resulting plasmids, the gene was under the control of the constitutive cauliflower mosaic virus 35S promoter and NOS terminator. The plasmids were further digested with AscI/ PacI, and the fragment obtained was inserted into AscI/PacI site in the expression vectors pBinPLUS (van Engelen et al., 1995) and pMF2 (Schaart et al., 2010) to create pBinPLUS:*DFR*, pMF2:*ANS* and pBinPLUS:*MYB12* plasmids. Note that pBinPLUS contains kanamycin selection marker, while pMF2 contains hygromycin selection marker.

For *F3'H*, we were unable to PCR-amplify the gene from 'Gran Tourismo'. Therefore, a synthetic *F3'H* gene was synthesized from *Lilium speciosum* having red tepals (AB911306.1). The *F3'H* sequence was designed compatible for the GATEWAY system, and then cloned into pGWB502Ω (Nakagawa et al., 2007) using LR-Clonase enzyme II (Invitrogen). In the resulting plasmid pGWB502Ω:*F3'H*, the gene was under the control of double constitutive cauliflower mosaic virus 35S promoter and NOS terminator with hygromycin selection. Finally, all the resultant plasmids were introduced into *A. tumefaciens* strain AGL0 (Lazo et al., 1991) by electroporation method.

The construction of pBinPLUS:*FBP1:ROS1+DEL* containing *ROS1* and *DEL* transcription factors from snapdragon under a control of a flower specific promoter *FBP1* from petunia is described in **Chapter 3**.

Transient gene expression in *Lilium* tepals

To determine the functionality and the ability of the genes-of-choice to upregulate (or suppress) the expression of the endogenous anthocyanin biosynthesis genes, we transiently expressed the *F3'H*, *DFR*, *ANS*, *MYB12* and *FBP1:ROS1+DEL* constructs in tepals of the Oriental hybrid lily cv. 'Perth'.

A single colony of *Agrobacterium* was inoculated in 10 mL liquid Luria-Bertani broth (LB) medium containing 50 mg/L kanamycin and 50 mg/L rifampicin and was grown overnight at 28˚C in a shaker (150 rpm) to an OD_{600} ± 1.0. In order to keep the culture in exponential growth phase, it was diluted 1:10 after 24 hours. The following day, *Agrobacterium* was pelleted by centrifugation at 3000 rpm for 15 mins, the supernatant was discarded, and the pellet was washed once with sterile demineralized water. After washing, the pelleted *Agrobacterium* was resuspended in MMA medium (20 g/L sucrose, 5 g/L MS salts (no vitamins), 1.95 g/L MES, 100 µM acetosyringone, pH was adjusted to 5.6 with NaOH, and the medium was filter sterilized) at bacterial density OD_{600} 1.0 (Yasmin and Debener, 2010; Pinthong et al., 2014). Paclobutrazol (PBZ) at a final concentration of 10 µM was added to the infiltration buffer to enhance transformation efficiency (Hasan Nudin et al., 2015). *Agrobacterium* suspensions were then incubated at room temperature for 1-3 hours for acclimatization before use. The three outer tepals of an intact and closed flower bud (i.e. before anthesis) were pricked in the middle by a sterile syringe-needle to facilitate penetration, and subsequently infiltrated with 0.5 mL of *Agrobacterium* suspension using a 1 mL needleless syringe. Among the three outer tepals, two were infiltrated with *Agrobacterium* containing the constructs and one was infiltrated with *Agrobacterium* containing pCAMBIA+GUS (Cambia, Australia) as a control. The histochemical *GUS* assay was performed according to (Jefferson, 1987). For each construct, three flowers from six plants which made a total of 18 flowers were infiltrated. The infiltrated plants were kept in a greenhouse condition with 16/8 h light-dark photoperiod, temperature at 21˚C and relative humidity at 60%. At 6 days after the infiltration, about 3 cm x 3 cm of infiltrated areas in tepals were harvested for phenotypic observation, photography, anthocyanin measurements and RNA isolation. The samples were pooled for each treatment, immediately frozen in liquid nitrogen and kept in a -80˚C freezer until use.

Next, to supplement all anthocyanin gene(s) that are lacking in 'Rialto' and 'Lincoln', the different constructs were introduced separately as well as in different combinations using the method described for syringe infiltration above. *Agrobacterium* mixtures aimed at combining constructs were prepared by adding the different *Agrobacterium* suspensions in equal volumes (1:1 or 1:1:1 or more).

Determination of anthocyanin content

Anthocyanins and related compounds were extracted from lily tepals (100 mg dry weight) with 5 mL of extraction solvent containing 1% (v/v) hydrochloric acid (HCl) and 0.1% (v/v) butyl hydroxyl anisol (BHA) in methanol. Three replicates of each cultivar in each stage were used for extraction. The extraction mixture was sonicated for 15 min in an ultrasonic cleaner (VWR International, 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 2N HCl was heated in a heating block set for 120 min at 99ºC.

The hydrolysed samples 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% B at 0-35 min, 28-75% B at 35-37 min, 75% B at 37-40 min, 75-5% B at 40-42 min, 5% at 42-50 min. Chromatograms were acquired at 512 nm (anthocyanins), and five concentration levels (0.01, 0.025, 0.05, 0.1 and 0.2 mg/mL) of cyanidin chloride were used to make calibration curve (Fig. 2). Quantification of anthocyanins was performed by correlating the chromatographic peak area with concentrations in accordance with the calibration curve of the corresponding external standard.

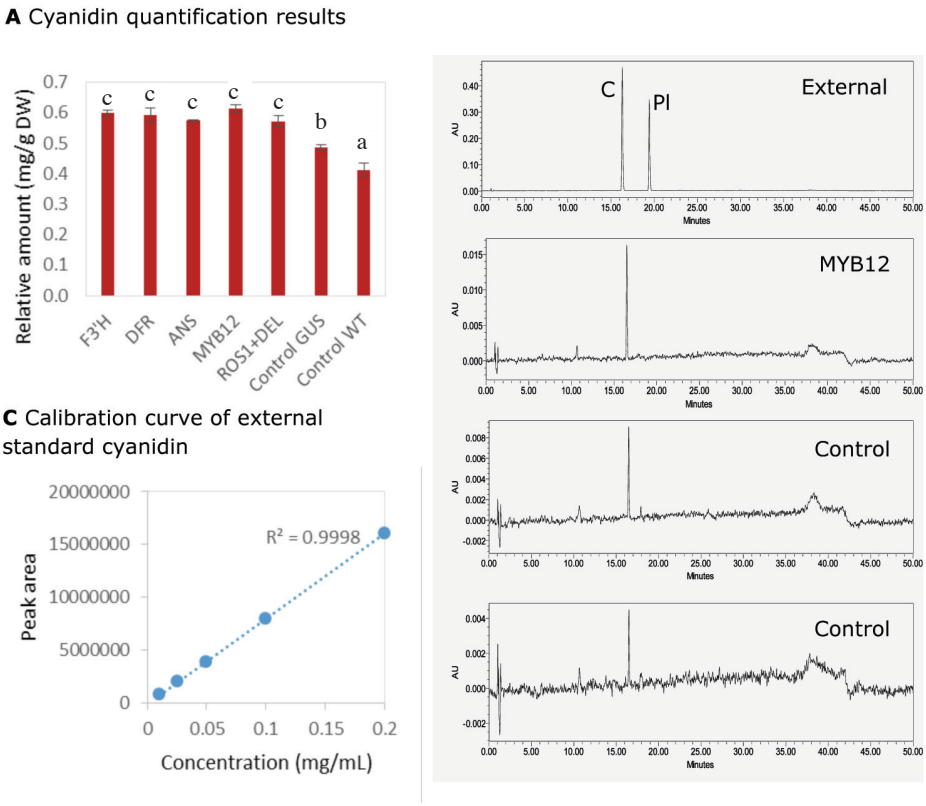

Fig. 2. Biochemical of 'Perth' phenotype. (A) Relative quantification of cyanidin (mg/g dry weight) measured in hydrolysed samples of 'Perth' infiltrated tepals. (B) Representations of selected HPLC results. The examples show the chromatograms obtained from HPLC analysis (detection wavelength of anthocyanins = 512 nm). The external standards, cyanidin (Cy) and pelargonidin (Pl) are labelled. 'Perth' tepals infiltrated with *MYB12*, *GUS* and wild type (WT) tepals show only cyanidin accumulated. (C) The areas of the cyanidin peaks were normalized according to the calibration curve of the corresponding external standard cyanidin (0.01, 0.025, 0.05, 0.1 and 0.2 mg/mL) resulting in relative cyanidin amounts.

Quantitative real-time PCR (qPCR)

To investigate the transcription levels of anthocyanin structural genes (*F3'H, DFR* and *ANS*) and *MYB12* transcription factor gene in the three lily cultivars 'Perth', 'Rialto' and 'Lincoln', quantitative real-time PCR (qPCR) was performed using CFX96TM real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA). The final reaction volume (20 µL) contained 1 µL of first-strand cDNA, 10 µL 2X iQ SYBR GREEN super mix 2X DNA fluorescent dye (Bio-Rad Laboratories, Inc., Hercules, CA, #172-5006 CUST), 2 µL of

each forward and reverse primers (3 µM), and 5 µL miliQ water. Cycling conditions were: preheating at 95˚C for 3 min, 40 cycles of 95˚C for 15 sec 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 sec. The gene specific primers were designed by Primer3Plus program (http://primer3plus.com/) and some were extracted from literatures (Table 1). Note that the primers used in this study were not unique for the endogenous genes but can also amplify the introduced transgenes. *Lilium Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* and *Arabidopsis Actin* mRNA were 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 1. The relative gene expression (RGE) was determined based on the $2^{-\delta\delta Ct}$ calculation method (Livak and Schmittgen, 2001). Three biological replicates per treatment were used in the analysis, and each replicate was analysed in duplo.

Table 1. List of primers used for qPCR and their efficiency.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Primer efficiency
LhGAPDH	CTACTGTGCACGCCATCACT	ACACATCGACAGTGGGAACA	1.963
AtACTIN	GGCGATGAAGCTCAATCCAAACG	GGTCACGACCAGCAAGATCAAGACG	1.846
LsF3'H	ACGCACGACACAAACTTCAG	CGAGTGCTTTCTGGGAAAAG	1.918
LhDFR	ATATGCCATCCCCCAAAAGT	GCAACCCAAATCCAGTTCAT	1.912
LhANS	GGGGGAATGGATGACCTACT	GTTGGTGAGGAGGAAGGTGA	1.867
LhMYB12	GGGTGAAGCTGAACCAAAAA	GTCCCATTGGAGAATTGCAT	1.932

Lh, Ls, At indicate the primers were designed based on the *L. hybrid, L. speciosum, A. thaliana* sequences, respectively

Statistical data analysis

Data was subjected to one-way analysis of variance (ANOVA) and means were compared using Duncan's multiple range test at $P<0.05$. The statistical analysis was performed by SPSS version 22.

Complementation analysis in *Arabidopsis* mutants

To further confirm the functionality of the genes, the *F3'H, DFR* and *ANS* were introduced to complement *Arabidopsis* mutants tt7-3, tt3-1 and tds4-2, respectively. About 5-week-old *Arabidopsis* plants with few mature flowers were transformed by floral dip (Clough and Bent, 1998). T1 seeds were sterilized with 2% (w/v) sodium hypochlorite (NaClO) for 15 minutes, followed by rinsing three times with sterile distilled water for 10 minutes. The seeds were selected and germinated on ½ MS 15 medium (2.2 g/L Murashige and Skoog medium

(MS) salts and vitamins (Duchefa, Haarlem, The Netherlands), 15 g/L sucrose, 0.7% (w/v) micro agar, pH adjusted to 5.8) containing 25 mg/L kanamycin or hygromycin. The seeds were kept in dark at 4˚C for 4 days before grown in a growth chamber at 22˚C with white light (Philips, TL-D 36W/840). After 3 weeks of culture on selection medium, T1 transgenic seedlings were transferred to soil to set T2 seeds. T2 seeds were then harvested and cultured on ½ MS 15 medium containing 25 mg/L kanamycin or hygromycin. After 3 weeks, T2 transgenic seedlings were used for qPCR to confirm expression of exogenous *F3'H, DFR* and *ANS* genes.

Results

Transient expression in 'Perth' tepals

Changes in anthocyanin content (Fig. 2) and in gene expression (Fig. 3) were analysed in 'Perth' tepals. Note that the expression of the introduced gene as it is measured is always a combination of the expression of the endogenous gene and the introduced transgene (indicated as 'combination'). Transient expression of *F3'H* from the red *L. speciosum* into pink 'Perth' significantly increased the expression level of *F3'H* (combination) at 40-fold higher than the wild type 'Perth'. The expression of endogenous *DFR* and *ANS* genes were also increased at about 18-fold and 11-fold, respectively, and the levels were significantly different from the ones measured after introduction of the *GUS* gene as control. As a result, cyanidin accumulation was increased to 0.60 mg/g (dry weight) compared to the control wild type of about 0.41 mg/g and the control *GUS* of about 0.49 mg/g. Next, infiltration of *DFR* from 'Gran Tourismo' showed a little stimulation of 'Perth' *F3'H*, but the expression levels of *DFR* (combination) and *ANS* were increased to a comparable level as after infiltration with *F3'H*. This was supported by an increase in anthocyanin content; *DFR*-infiltrated tepals accumulated 0.59 mg/g cyanidin. Introduction of *ANS* from 'Gran Tourismo' also showed little stimulation on 'Perth' *F3'H*, but higher *DFR* (25-fold) and *ANS* (16-fold; combination) transcript levels were observed. However, cyanidin content was increased to the same level as after infiltration by *DFR*, to 0.57 mg/g. For the transcription factors, transient expression of 'Gran Tourismo' *MYB12* did highly increase the expression of endogenous *DFR* to about 33-fold change and *ANS* to about 20-fold change, resulting in deeper pink colouration measured at 0.61 mg/g cyanidin level. It also positively influenced expression of the *MYB12* (combination) but only showed a slight stimulation of *F3'H* in 'Perth'. On the other hand, the introduction of the *Antirrhinum* transcription factor genes *ROS1+DEL* did not show the same prominent stimulatory effect on expression of *F3'H, DFR* nor on *MYB12*. Still as a result, the level of *ANS* was induced 16-fold which was at par with the *ANS*-expressing tepals, and the cyanidin content was increased to 0.57 mg/g.

Based on the transient expression assay in 'Perth' tepals with an intact anthocyanin biosynthetic pathway (Fig. 4), all the constructs containing a target gene (*F3'H, DFR, ANS, MYB12* and *ROS1+DEL*) seemed to be functional in inducing anthocyanin formation, in most cases through stimulation of endogenous *DFR* and *ANS*.

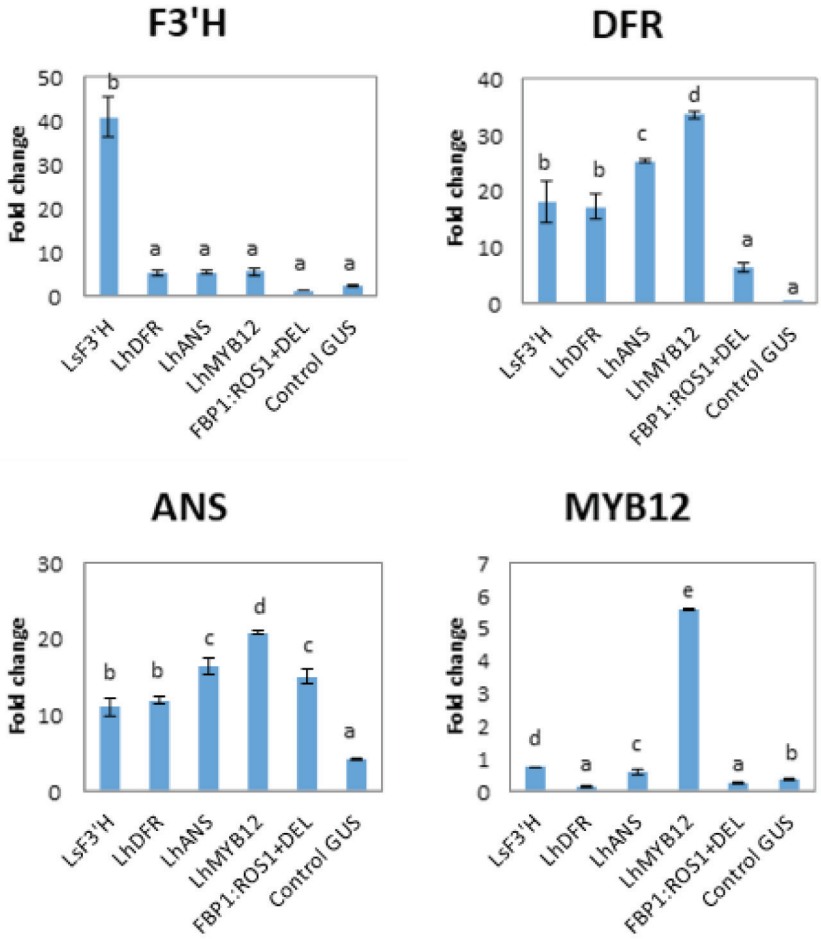

Fig. 3. Relative gene expression patterns in 'Perth' tepals infiltrated with *LsF3'H, LhDFR, LhANS, LhMYB12, FBP1:ROS1+DEL,* and *GUS* as a control. Error bars indicate the standard error of the mean ± SE of three replicate measurements. *Ls* = *L. speciosum*, *Lh* = *L. hybrid.*

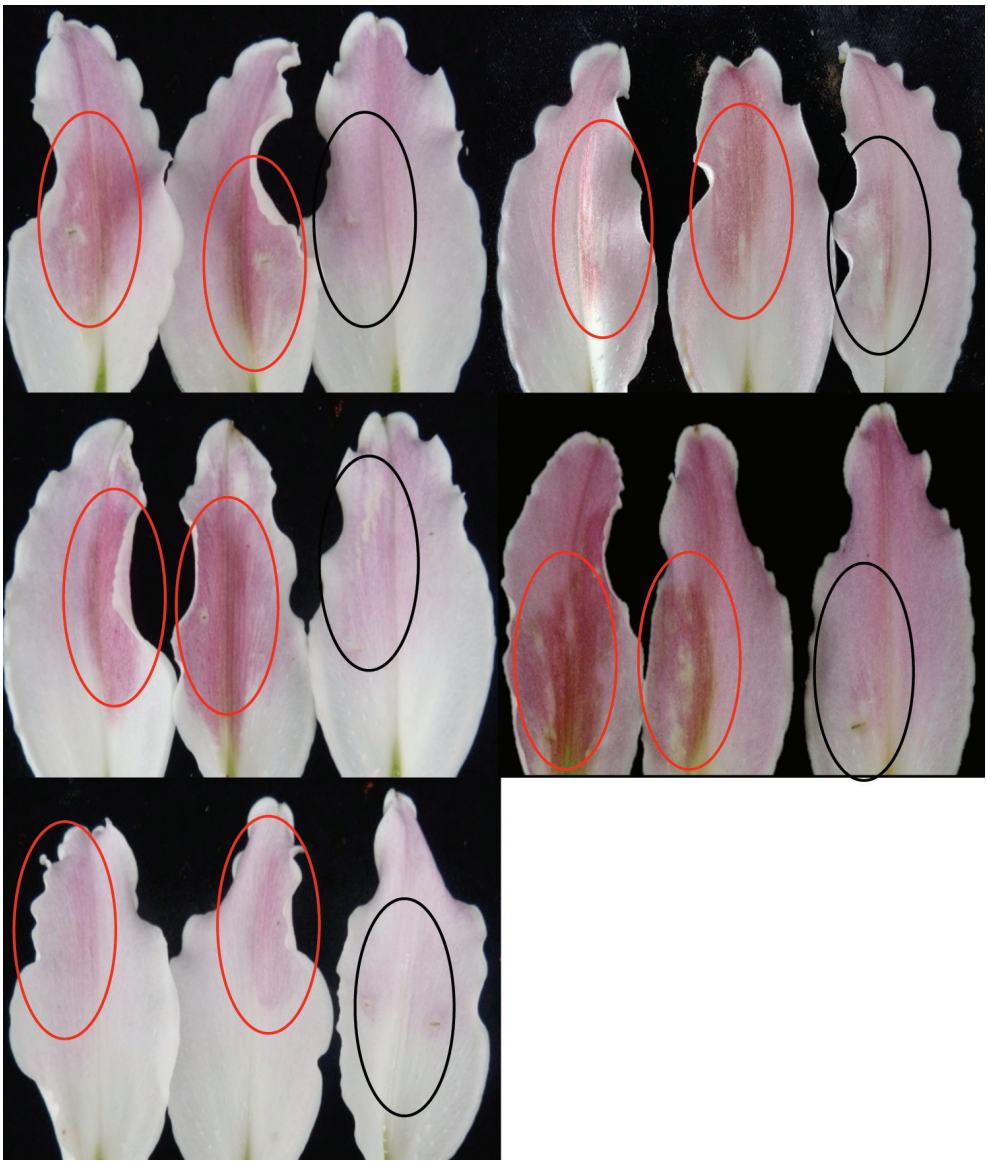

Fig. 4. Anthocyanin accumulation showing deeper pink colouration at the infiltrated area of 'Perth' outer tepals. In each flower, two outer tepals were infiltrated with (A) *LsF3'H*, (B) *LhDFR*, (C) *LhANS*, (D) *LhMYB12*, (E) *ROS1+DEL* (shown in red circles) and one outer tepal was infiltrated with *GUS* as a positive control of *Agrobacterium* (shown in black circles).

Transient expression in 'Rialto' and 'Lincoln' tepals

In 'Rialto', introducing *DFR* from 'Gran Tourismo' should produce colour if that *MYB12* is functional. On the other hand, if the W-to-L amino acid change in *MYB12* has disrupted its function as previously mentioned (Yamagishi, 2011; Yamagishi et al., 2012), introducing a functional *MYB12* from 'Gran Tourismo' should be able to restore the gene function. The expression levels of *F3'H, DFR, ANS* and *MYB12* in 'Rialto' tepals were measured (Fig. 5). Because no treatment whatsoever showed any increase in 'Rialto' endogenous *DFR* expression, these data are not presented in Fig. 5. A stimulatory effect on *MYB12* expression was only observed after introducing *DFR*. Introducing the 'Gran Tourismo' *MYB12* gene significantly increased the expression level of *MYB12* (combination) approximately 2-fold higher than the non-infiltrated control and also significantly increased endogenous *F3'H* and *ANS* approximately 6-fold each. However, the expression levels were not stimulated to the same extent when both genes (*DFR* and *MYB12*) were co-infiltrated simultaneously. To induce the production of anthocyanins in 'Rialto', co-infiltrations of *F3'H+DFR+ANS* plus either *MYB12* or *FBP1:ROS1+DEL* were attempted. However, neither expression of *DFR* nor anthocyanin formation was detected in 'Rialto' after the introduction of the target genes in different infiltration schemes.

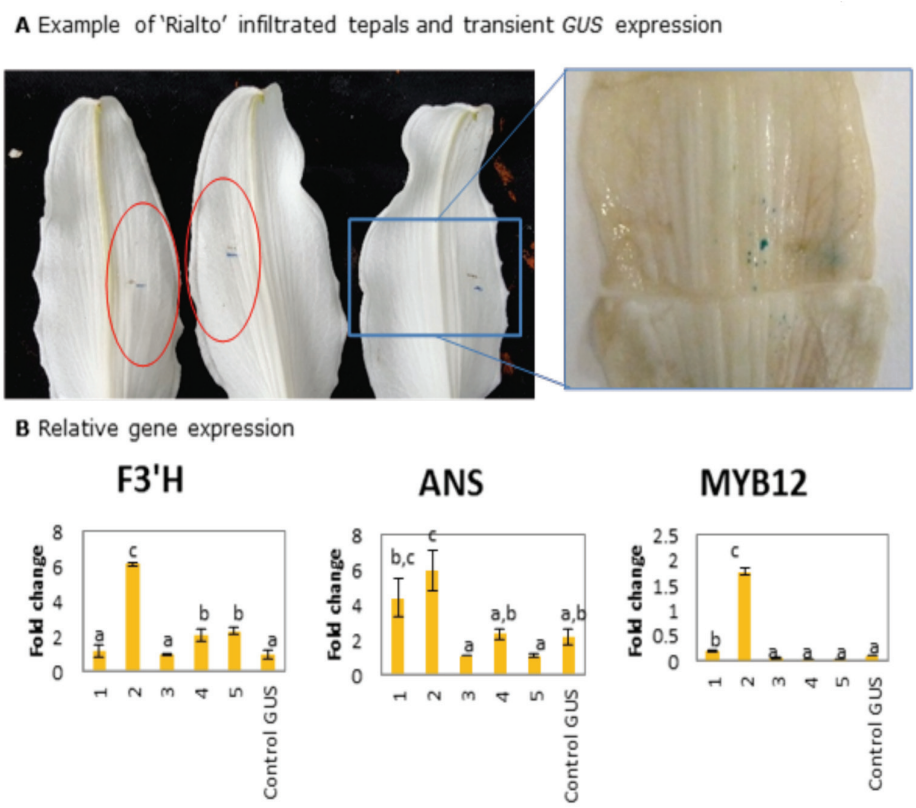

Fig. 5. Representation of 'Rialto' infiltrated tepals and relative gene expression. (A) No anthocyanin pigmentation was seen at the infiltrated area (shown in red circles) and *GUS* expression was seen at the infiltrated area of *GUS* control tepal. (B) Relative gene expression patterns in 'Rialto' tepals infiltrated with 1) *LhDFR*, 2) *LhMYB12*, 3*) LhDFR + LhMYB12*, 4) *LsF3'H+LhDFR + LhANS + LhMYB12*, 5) *LsF3'H + LhDFR + LhANS + FBP1:ROS1+DEL*. *GUS* was used as a control. Error bars indicate the standard error of the mean ± SE of three replicate measurements. An example of infiltrated 'Rialto' tepals is shown at the right bottom, and marked with red arrows. *Ls = L. speciosum*, *Lh = L. hybrid.*

For 'Lincoln', it was previously found that *F3'H, DFR* and *bHLH2* were absent and not expressed, which was likely responsible for the white colour of the tepals. Together with that, *ANS* was found to be lowly expressed. To test this hypothesis, we transiently co-expressed *F3'H, DFR, ANS, MYB12* and *FBP1:ROS1+DEL* in four infiltration schemes into 'Lincoln' tepals. The expression levels of *F3'H, DFR, MYB12* and *ANS* in 'Lincoln' tepals were measured (Fig. 6). Similar to 'Rialto', neither expression of *DFR* nor anthocyanin formation was detected in 'Lincoln' tepals after the introductions of different combinations of constructs. Introducing *F3'H+DFR* did not stimulate the expression of *F3'H*, *ANS* and *MYB12* at a significant level. Co-infiltration of *F3'H+DFR+ANS* stimulated the expression

level of *ANS* (3.5-fold) higher than without the inclusion of exogenous *ANS*. It was hoped that *DEL* (a *bHLH*-type) transcription factor from snapdragon could restore the function of *bHLH2* in 'Lincoln'. So, inclusion of *FBP1:ROS1+DEL* together with *F3'H+DFR+ANS* has significantly stimulated the expression levels of *F3'H* up to 0.15-fold and *ANS* up to 18-fold. Instead of adding *FBP1:ROS1+DEL,* adding *MYB12* together with *F3'H+DFR+ANS* did not enhance the expression levels of *F3'H* and *ANS,* while *MYB12* expression itself (combination) was increased significantly. Based on the results, it seems that *DFR* was the ultimate blocking step in the anthocyanin biosynthetic pathway of 'Rialto' and 'Lincoln' tepals. Moreover, 'Lincoln' really needs a *bHLH2*-type transcription factor to stimulate the expression of anthocyanin structural genes.

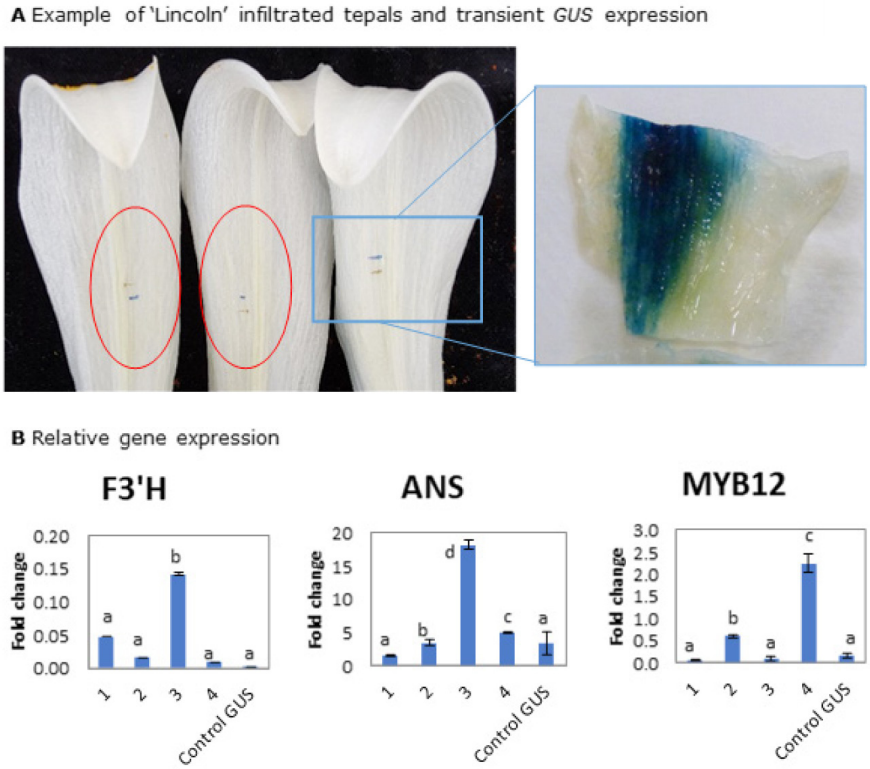

Fig. 6. Representation of 'Lincoln' infiltrated tepals and relative gene expression. (A) No anthocyanin pigmentation was seen at the infiltrated area (shown in red circles) and *GUS* expression was seen at the infiltrated area of *GUS* control tepal. (B) Relative gene expression patterns in 'Lincoln' tepals infiltrated with 1) *LsF3'H* + *LhDFR*, 2) *LsF3'H* + *LhDFR* + *LhANS,* 3) *LsF3'H* + *LhDFR* + *LhANS* + *FBP1:ROS1+DEL* and 4) *LsF3'H* + *LhDFR* + *LhANS* + *LhMYB12. GUS* was used as a control. Error bars indicate the standard error of the mean ± SE of three replicate measurements. An example of infiltrated 'Lincoln' tepals is shown at the right bottom, and marked with red arrows. *Ls = L. speciosum*, *Lh = L. hybrid.*

Stable expression of *F3'H, DFR* and *ANS* in *Arabidopsis* mutants

As mentioned above, introducing *DFR* from 'Gran Tourismo' could induce anthocyanin accumulation in 'Perth' tepals, but could not restore the function of *DFR* in the white-flowered 'Rialto' and 'Lincoln'. In order to further confirm the potential functionalities of *F3'H*, *DFR* and *ANS,* we introduced the genes into *Arabidopsis* mutants tt7-3, tt3-1 and tds4-2, respectively. Phenotypic observation showed that introduction of *F3'H* and *ANS* did not restore anthocyanin pigmentation in the seed coats, but produced purple colouration in the hypocotyls of the T2 transgenic seedlings (Figs. 7 and 9). However, introduction of *DFR* failed to restore anthocyanin pigmentation in both seed coats and hypocotyls of the tt3-1 mutant (Fig. 8). The presence and expression of each introduced gene in the transgenic seedlings was further confirmed by qPCR and the results were visualized on agarose gel (Figs. 7-9). No amplified fragments were observed in the wild type and mutant seedlings, whereas amplified fragments of the expected size were observed in the transgenic seedlings expressing *F3'H* and *ANS*. In parallel with the phenotypic observation, no fragment was amplified from the transgenic seedlings that have been transformed with the *DFR* construct and germinated on kanamycin containing medium.

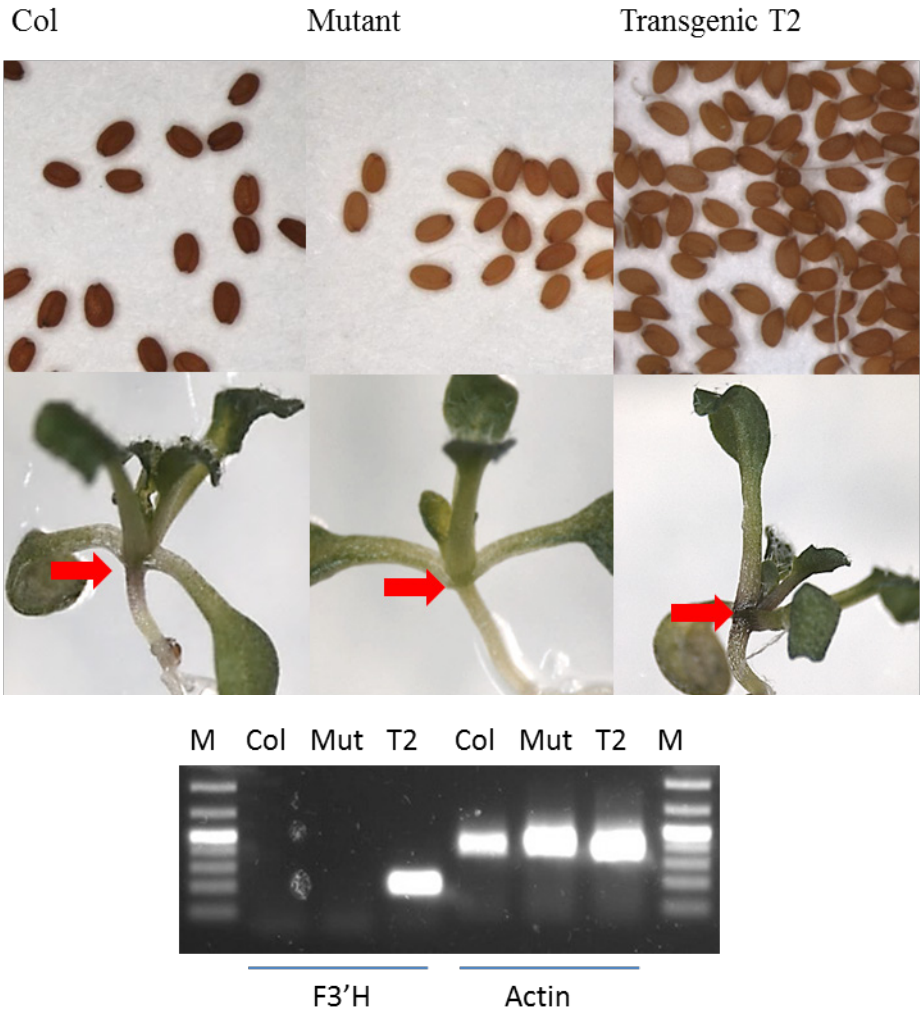

Fig. 7. Complementation of *Arabidopsis* tt7-3 mutant expressing *LsF3'H*. Phenotype of the wild type Col, tt7-3 mutant and T2 transgenic seed coats and seedlings. Expression analysis of *LsF3'H* by qPCR in the wild type Col, tt7-3 mutant and a representative T2 transgenic seedling.

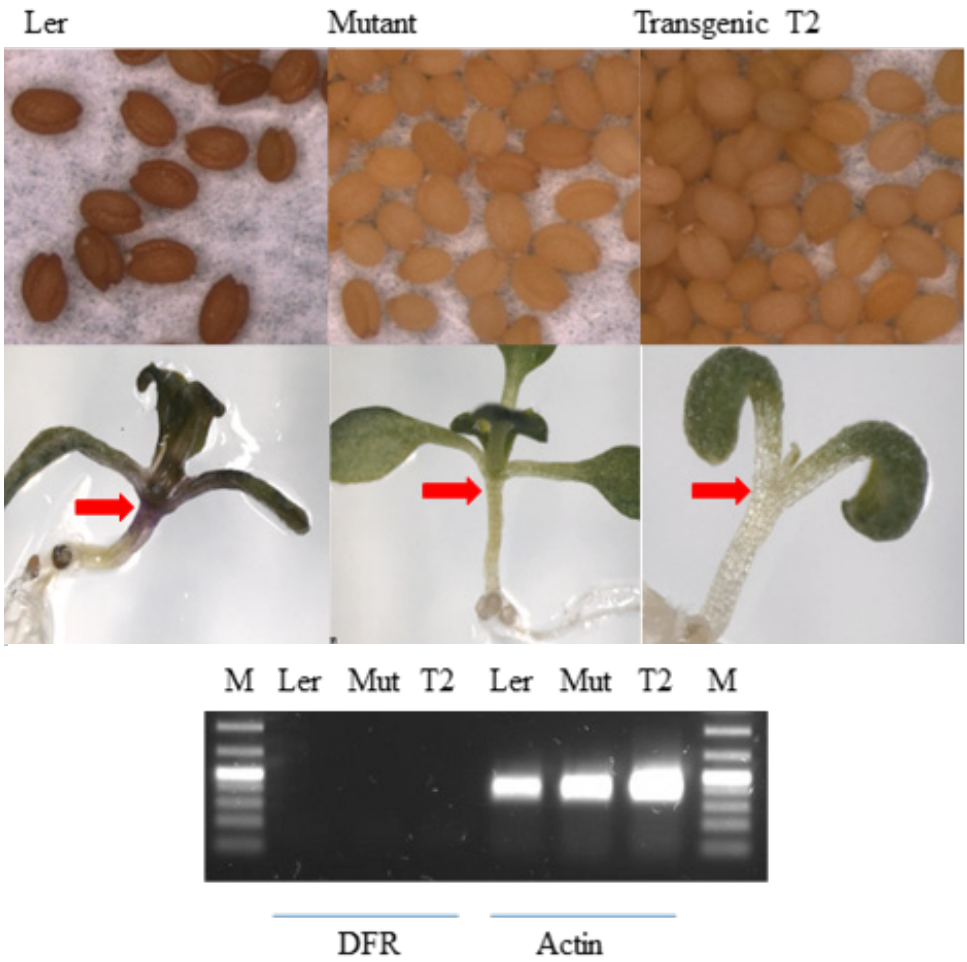

Fig. 8. Lack of complementation of *Arabidopsis* tt3-1 mutant after introducing *LhDFR*. Phenotype of the wild type Ler, tt3-1 mutant and T2 transgenic seed coats and seedlings. Expression analysis of *LhDFR* by qPCR in the wild type Ler, tt3-1 mutant and a representative T2 transgenic seedling.

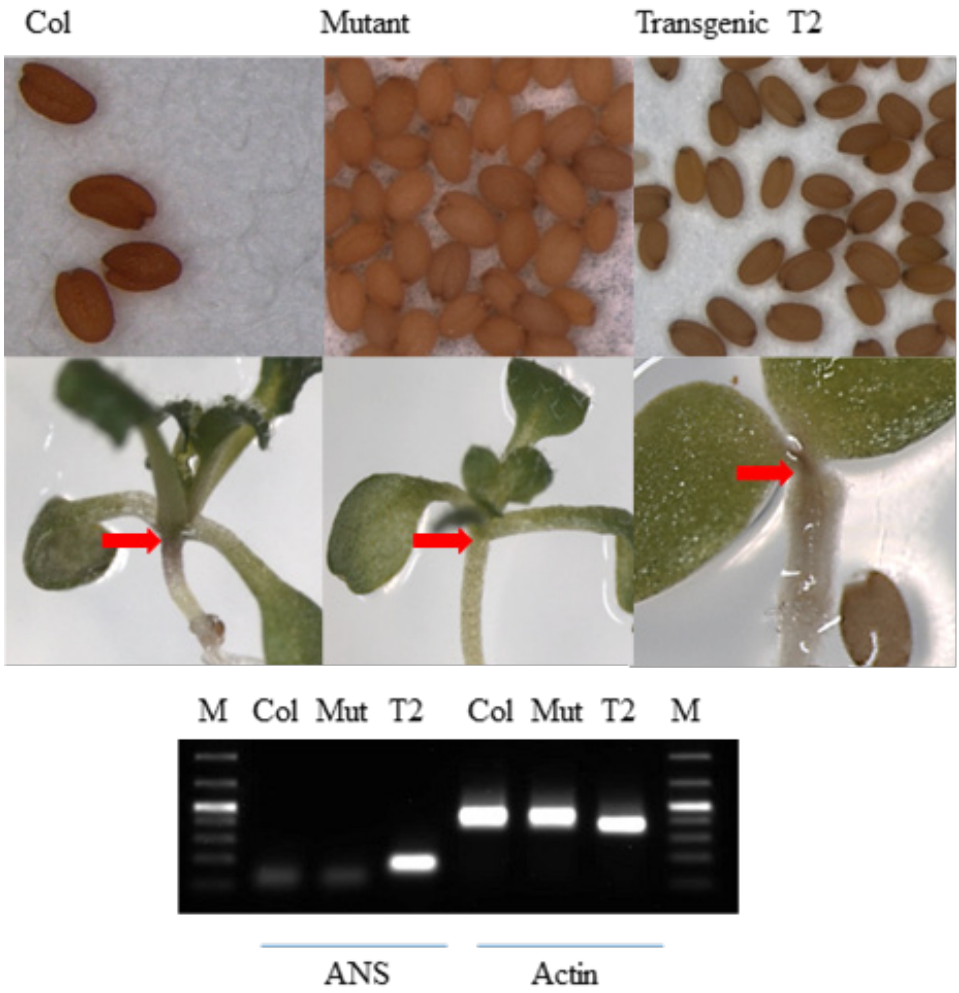

Fig. 9. Complementation of *Arabidopsis* tds4-2 mutant expressing *LhANS.* Phenotype of the wild type Col, tds4-2 mutant and T2 transgenic seed coats and seedlings. Expression analysis of *LhANS* by qPCR in the wild type Col, tds4-2 mutant and a representative T2 transgenic seedling.

Discussion

The increasingly rapid isolation and identification of plant genes have broadened the scope of efforts aimed to produce novel traits in floriculture such as flower colour. Anthocyanins are the most common pigments contributing to attractive colours and antioxidants in plant organs (Schreiber et al., 2012). Appropriate genes encoding anthocyanin biosynthetic enzymes have been isolated and identified from different plants, including lilies (Nakatsuka et al., 2003; Liu et al., 2011; Lai et al., 2012; Suzuki et al., 2015). However,

successful flower colour modification is not only depending on identification of appropriate genes, but frequently require introduction of multiple genes. The efficient introduction of multiple genes into a host plant has become a common technical limitation (Tanaka et al., 2005). Moreover, lilies are among the monocotyledonous species considered generally to be more recalcitrant to *Agrobacterium tumefaciens* transformation and their regeneration frequency varies between genotypes (Wang et al., 2012). Consequently, the study of gene function in lilies was hampered by the low transformation efficiency, variation in regeneration ability and the long time span needed for the generation of transgenic flowering plants with desired traits. Therefore, a simple, transient gene expression assay which allows rapid evaluation of the gene function without having to go through the entire transformation process is desired. Transient agroinfiltration methods have been used to introduce one target gene or combinations of multiple genes directly into floral tissues such as in petunia, tobacco (Shang et al., 2007), rose (Yasmin and Debener, 2010) and orchid (Pinthong et al., 2014).

Anthocyanin biosynthesis in lilies is mainly regulated by the *MYB12* transcription factor through activation of anthocyanin structural genes (Yamagishi and Nakatsuka, 2017). In this study, we analysed the potential functions of anthocyanin structural genes *F3'H, DFR, ANS* and transcription factor *MYB12* in stimulating anthocyanin biosynthesis in lilies. The *DFR, ANS* and *MYB12* genes used for transformation were isolated from 'Gran Tourismo' having red tepals. Contrary to our success in amplifying those genes, we were unable to amplify the *F3'H* from 'Gran Tourismo'. Using several different PCR programs, different annealing temperatures and different primer pairs (Table S1), eventually, only non-reproducible and aspecific fragments were observed (data not shown). Gene duplication and the presence of short $CT_{(n)}$ repeats in the coding region of *F3'H* (Han et al., 2010) might have interfered with isolation and could also explain the aspecific fragments. Therefore, it was decided to synthesize a synthetic *F3'H* gene based on the *LsF3'H*-rr sequence (AB911306.1) from *Lilium speciosum*.

Introducing *F3'H* into 'Perth' tepals increased the accumulation of *F3'H* mRNA and increased expression of the later genes, *DFR* and *ANS*, and subsequently increased cyanidin content (Figs. 2-4). The functionality of the *F3'H* gene was then confirmed by complementing the *Arabidopsis* mutant tt7-3 (Fig. 7). Similarly to *F3'H*, the introduced *ANS* stimulated the *ANS* and *DFR* expression levels, resulting in more cyanidin in 'Perth' tepals compared to the non-infiltrated tepals. The function of *ANS* was also confirmed by anthocyanin restoration in the hypocotyl of the *Arabidopsis* mutant tds4-2 (Fig. 9). Introducing *DFR* did increase *DFR* expression and anthocyanin content in 'Perth' tepals. However, checking the sequence of the amplified fragment responsible for the increased *DFR* levels in 'Perth' tepals showed that the gene copy originated from the resident 'Perth' *DFR*. Moreover, complementing studies in *Arabidopsis* mutant tt3-1 showed some green transgenic *Arabidopsis* survived on kanamycin media, indicating that the kanamycin resistant *NPTII* gene is active, still the anthocyanin

pigmentation was not restored. This indicated that the introduced *DFR* gene copy from 'Gran Tourismo' might not be active in *Arabidopsis*, resulting in absence of a fragment after qPCR (Fig. 8). Based on the combined results obtained with *DFR*, there was no direct evidence that the isolated *DFR* gene copy from 'Gran Tourismo' was expressed. This could be due to a construction error when making the vector, however, we did first sequence the *DFR* construct produced in *E. coli* as a control and it looked o.k. and in addition, we did observe an effect after infiltration on colour and expression of the endogenous gene copy. Still, we found that expression of endogenous *DFR* could be easily stimulated by several genes and perhaps low expression (below detection) of the introduced *DFR* because of a faulty construction still stimulated endogenous *DFR* in 'Perth' but did not complement the mutation in *Arabidopsis.*

Wang et al., (2013) argued that oxidative stress caused by *Agrobacterium* could also influenced anthocyanin fluctuation and produced false positive transgenic plants. Indeed, a mock infiltration with an *Agrobacterium* suspension containing pCAMBIA+GUS (Cambia, Australia) seemed to have little influence on anthocyanin accumulation (Fig. 2) and gene expressions (Fig. 3) in our transient expression assay, but the levels were always lower than that induced by the introduced genes.

For the transcription factors, transient expression of 'Gran Tourismo' *MYB12* highly increased the expression levels of *DFR* and *ANS*, resulting in the highest cyanidin accumulation which was equal to the other constructs. Nevertheless, *MYB12* showed only little stimulation of *F3'H* in 'Perth' tepals. The result was in agreement with Lai and coworkers (2012), where by *MYB12* in Asiatic hybrid lily could regulate both early and late structural genes, but could not regulate all the genes from *CHS* to *ANS*. These observations showed that among the monocotyledonous plants, lily *MYB12* is different from maize *C1* and *Pl* which were able to regulate all the anthocyanin structural genes (Cone et al., 1993).

Although expression of a functional lily *MYB12* alone could induce anthocyanin in *N. benthamiana* leaves (Suzuki et al., 2015), *MYB12* usually interacts with *bHLH2* to activate the transcription of anthocyanin structural genes (Yamagishi et al., 2010; Lai et al., 2012). Based on the successful expression of *ROS1* and *DEL* transcription factors in *N. benthamiana* and 'Perth' tepals demonstrated in our previous experiments (**Chapter 3**), it was hoped that snapdragon *DEL* could complement the *bHLH2* lacking in 'Lincoln'. Co-expression of multiple genes, including *F3'H, DFR, ANS, MYB12* and *FBP1:ROS1+DEL* has been attempted to manipulate flower colours in 'Lincoln' and 'Rialto', but unfortunately, none of the infiltration schemes worked in neither of the two cultivars. The expression levels of *F3'H, ANS* and *MYB12* could be upregulated, but not expression of *DFR*. Still as a result, co-infiltration of *F3'H+DFR+ANS* together with *FBP1:ROS1+DEL* in 'Lincoln' stimulated *ANS* compared to that with *MYB12* alone, indicating that the presence of *bHLH*-type transcription factor is necessary in 'Lincoln'.

A number of infiltration schemes combining *F3'H+ANS+FBP1:ROS1+DEL* or *F3'H+ANS+MYB12* together with a functional *DFR* gene either from *Gerbera DFR1, DFR2, DFR3* or *Petunia DFRa* also failed to bring any phenotypic difference in 'Rialto' and 'Lincoln' (data not shown). The failure might be caused by a constraint in expecting no less than four different *Agrobacterium* strains to transfer successfully their T-DNA into the same cells at the same time. Therefore, combining the target genes into one T-DNA is recommended for future research.

Taken together, this study demonstrates that the isolated copies of *F3'H* and *ANS* from lily were two functional genes that proved able to induce anthocyanin biosynthesis in the Oriental hybrid lily cv. 'Perth' and restored the gene function in *Arabidopsis* mutants. Testing compensation of *Arabidopsis* mutant and sequencing the increased *DFR* levels in 'Perth' introduced by *DFR* from 'Gran Tourismo' showed that the gene copy was not expressed. *MYB12* from 'Gran Tourismo' induced anthocyanin accumulation in 'Perth' tepals through activation of *DFR* and *ANS*. However, co-expression of multiple genes failed to induce anthocyanin biosynthesis in the white-flowered 'Rialto' and 'Lincoln' due to the complexity of anthocyanin biosynthetic pathway. The absence of a functional *DFR* gene copy seemed to be the main bottleneck in the pathway of 'Rialto' and 'Lincoln'. Thus, introducing a functional and suitable *DFR* gene copy from lily or another ornamental crop remains to be investigated in order to produce novel flower colour in both cultivars. Nevertheless, the final visibility and stability of flower colour is generally a combination of several factors including the type of anthocyanin accumulating, modifications to the anthocyanidin molecule, co-pigmentation and vacuolar pH.

Acknowledgement

We are grateful to Prof. Teemu Teeri (University of Helsinki, Finland) for supplying *Gerbera* and *Petunia DFR* clones.

Table S1. List of primers used for cloning.

Primer names	Sequences from 5'-3'
GtDFR_full_F-SstI	GTCAGAGCTCATGGAGAATGCGAAAGGACCCGTGG
GtDFR_full_R-BglII	GTCAAGATCTTTACTGAAGAGCAACGGAGACTCGT
GtANS_full_F-NcoI	GTCACCATGGATGCCGACCGAGATCATGCCGTTGC
GtANS_full_R1-BglII	GTCAAGATCTTCACTTGAGAGAAGTGAAGTCCTCC
GtMYB12_F_NcoI	GTCACCATGGATGTTTCAAACGTTTATTGCCTCCG
GtMYB12_R_BglII	GTCAAGATCTTTATTCAACTTCGGAATCACTCCAA
GtF3'H_F-NcoI	GTCACCATGGATGGAAGCTCAACCTCTCCTCCTCC
GtF3'H_R1-SmaI	GTCACCCCGGGCTAGAGTTTCTCATTCTTCCCATAC
GtF3'H_R2-SmaI	GTCACCCCGGGCTAGAGTTTCTCATGCTTCCCATAT
GtF3'H_F-cacc	caccATGGAAGCTCAACCTCTCCT
GtF3'H_R1-cacc	CTAGAGTTTCTCATTCTTCC
GtF3'H_R2-cacc	CTAGAGTTTCTCATGCTTCC
GtF3'H_5'F-cacc	caccGACCAAACCGTCAGCCACCA
GtF3'H_F-cacc2	CACCATGGAAGCTCAACCTCTCCTCCTCCTYC
GtF3'H_R2-cacc2	CTAGAGTTTCTCATGCTTCCCATATGCC
LsF3'H_suzuki_F	CTCCTCCTCCTCCTCACCACCA
LsF3'H_suzuki_R	CTTCGCGAGCCTCGGCACT
CaMV35S_F	CTATCCTTCGCAAGACCCTTC
Tnos_R	TGCCAAATGTTTGAACGATC
M13_F	GTAAAACGACGGCCAG
M13_R	CAGGAAACAGCTATGAC

The restriction sites are underlined: SstI: GAGCTC, BglII: AGATCT, NcoI: CCATGG, SmaI: CCCGGG. Gt or Ls indicates the primers were designed based on the Oriental hybrid lily cultivar 'Gran Tourismo' and *L. speciosum* sequences, respectively.

CHAPTER 6

General Discussion

Floriculture is a very diverse sector that includes the production of floral crops such as cut flowers, flower bulbs, potted plants as well as garden plants. Global floriculture trade in 2015 was estimated at USD 55 billion, and the Netherlands serves as the main exporter for cut flowers, which amounted up to a 43% share (Fig. 1, Rabobank, 2016). Production increases in Colombia, Kenya, Ecuador and Ethiopia have been very high. Their combined cut flower exports have increased from 25% to 44% of the total world export production in 10 years' time (Rabobank, 2016). The most important cut flowers worldwide are rose, chrysanthemum, lily, gerbera, tulip and carnation (Chandler and Tanaka, 2007).

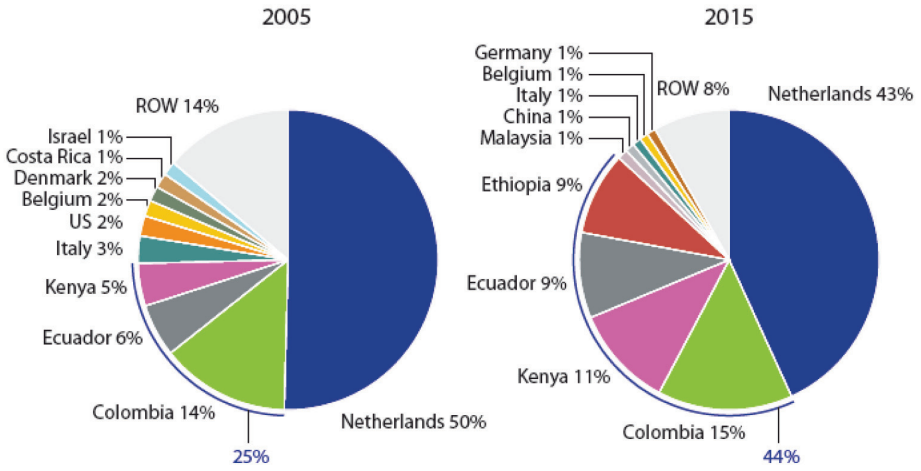

Fig. 1. Global exporters for cut flowers in 2005 vs. 2015. Source: UN-Comtrade, Royal FloraHolland, Rabobank, 2016. ROW = Rest of the World

Lilies (genus *Lilium*, family Liliaceae) are an important bulbous crop worldwide. There are more than 100 species that are widespread in the northern hemisphere, primarily in Asia, North America and Europe (Comber, 1949; De Jong, 1974; Lim et al., 2008). The species are taxonomically classified into seven sections (Comber, 1949). Hybrids within sections *Archelirion, Sinomartagon* and *Leucolirion* represent the economically most important groups. The Oriental hybrids are derived from interspecific crosses between *Lilium* species from the section *Archelirion,* and Asiatic hybrids are derived from interspecific crosses between *Lilium* species belonging to the section *Sinomartagon* (Leslie, 1982). The Asiatic and Oriental hybrids are the two most important groups in the world flower market (Lim and van Tuyl, 2006), and flower colour is their key attractive factor. The Asiatic hybrids display a huge variation in flower colours ranging from yellow, orange, pink, red and white, whilst the Oriental hybrids have big, showy flowers in pink, red and white (Yamagishi, 2013). On the other hand, Longiflorum hybrids derived from interspecific crosses between species belonging to the section *Leucolirion* only have white flowers.

The main aims of this thesis were to study anthocyanin as flower colour determinant in *Lilium* spp., and to alter the flower colours of lily via genetic modification. The entire research took a bottom-up strategy. The research started from the enhancement of *Agrobacterium*-mediated transformation efficiency using salicylic acid inhibitors to lower the plant's defence system and lipoic acid as antioxidant and stress alleviator in culture media during transformation (**Chapter 2**), by identifying the optimum concentration of the compounds in *Petunia hybrida* and *Nicotiana benthamiana* transformations. The best optimal concentration of SA inhibitors was then used to improve *Lilium* transformation via a flower agroinfiltration method (**Chapter 3**). Expression of *ROSEA1* (*ROS1*, a MYB-type) and *DELILA* (*DEL*, a bHLH-type) transcription factors from antirrhinum under control of a flower specific promoter, the *Floral Binding Protein 1* (*FBP1*) from petunia successfully stimulated anthocyanin biosynthesis in the flowers of *N. benthamiana* and the Oriental hybrid lily cv. 'Perth'. However, no pigmentation was induced in the white flowered *L. longiflorum* cv. 'Lincoln' and Oriental hybrid lily cv. 'Rialto', suggesting the absence or mutation of anthocyanin structural genes. This was followed by a molecular determination of the genetic background behind the white colouration and the mechanisms regulating flower colour in *Lilium* spp. (**Chapter 4**), and by the subsequent functional characterization of anthocyanin structural and transcription factor genes for flower colour modification in *Lilium* spp. (**Chapter 5**). Understanding the mechanisms underlying the colourless and coloured flowers of *Lilium* spp. should aid in the development of molecular tools for breeding of new originally white *Lilium* cultivars with altered flower colours. In this chapter, the general discussion, I would like to discuss the findings of this thesis in relation to recent findings published by other researchers.

Objectives in *Lilium* breeding

Most of the commercial *Lilium* hybrids nowadays are derived from interspecific hybridization between species belonging to the same section. Modern *Lilium* breeding focusses at inter-sectional hybridization and combining the three economically important groups: Asiatic (A), Oriental (O) and Longiflorum (L) hybrids. Although nowadays intersectional crosses are more and more aimed at combining resistances to biotic agents, such as insects, fungi and viruses, originally flower colour was the main reason for making such crosses. However, some important colours are still unavailable in certain important ornamental species. For example, *Lilium* spp. do not produce blue flowers because of the deficiency of the delphinidin-based key enzyme, F3'5'H (Martens et al., 2003). Moreover, *L. longiflorum* cultivars only exist in white flowers (Nakatsuka et al., 2007), so it is believed that creating any colour in *L. longiflorum* would be of potential commercial value.

A wide inter-sectional hybridization between Longiflorum x Asiatic hybrid or Longiflorum x Oriental hybrid lilies have been attempted in order to produce a new hybrid

combining the characters like flower shape of Longiflorum and flower colour of either Asiatic or Oriental hybrid lilies. However, improvement in *Lilium* breeding is often hampered by the limited genetic resources within the species or the section as well as by pre- and post-fertilization barriers after interspecific crosses (van Tuyl et al., 1991). These barriers frequently occur in the crosses between the two remotely related species, impairing hybrid formation and when leading to offspring, resulting in sterile F1 hybrids (Karlov et al., 1999).

Approaches to enhance *Lilium* transformation

A recent development in *Lilium* breeding is the introduction of genetic modification, which facilitates the introduction of commercially valuable genes directly into elite cultivars, adding new desired traits while preserving the elite, existing quality traits. The *Agrobacterium*-mediated and biolistic methods have been successfully used in *Lilium* transformation (Wang et al., 2012; Azadi et al., 2010; Kamo, 2008; Hoshi et al., 2005; 2004). However, it proved challenging to transform lily, because lily is thought to be recalcitrant to *Agrobacterium*, thus exhibiting low transformation efficiencies, and the efficiency varied between genotypes (Wang et al., 2012). Several optimization experiments have been done to enhance *Agrobacterium*-mediated efficiency in *Lilium* spp. such as wounding treatments prior to *Agrobacterium* inoculation (Hoshi et al., 2004), exclusion of $CaCl_2$, KH_2PO_4, KNO_3 and NH_4NO_3 (Azadi et al., 2010; Hoshi et al., 2005) and inclusion of 2-morpholinoethane-sulfonic acid (MES; Ogaki et al., 2008) in co-cultivation medium during transformation. Although Ogaki and coworkers (2008) have highlighted the importance of MES in the co-cultivation medium, a negative effect of bacterial overgrowth has been found for some sensitive cultivars in *Lilium* (Azadi et al., 2010). By employing these methods and the use of co-cultivation medium without MES, *Lilium* transformations were successfully achieved in several lily cultivars, but still at a low efficiency of 0.3 to 20.6% for transient transformation and 1.4% as the maximum for stable transformation (Wang et al., 2012).

One of the ways to improve *Agrobacterium*-mediated transformation efficiency is to make the plant host more susceptible to *Agrobacterium* infection. The use of salicylic acid (SA) as a signalling molecule to enhance tolerance to pathogens has been demonstrated in numerous experiments (An and Mou, 2011; Chen et al., 2009; Vlot et al., 2009; Zhu et al., 2014). In our study, we wanted the opposite effect and therefore, SA inhibitors were applied. In **Chapter 2**, we investigated the effect of adding SA and SA inhibitors (paclobutrazol, PBZ and 2-aminoindan-2-phosphonic acid, AIP) in the culture media during transformation of *Petunia hybrida* and *Nicotiana benthamiana*. The goal was to lower the plant's defence hence to facilitate in this way *Agrobacterium*-mediated T-DNA transfer. These two model species were selected for our experiments because their transformation protocols are well established, they are relatively easy to transform, and have short growth cycles which allow rapid evaluation of the results. Although the improvement of transformation efficiency in

these two model species has no practical importance, still it provides a good model. The results in **Chapter 2** demonstrated that SA is responsible for the plant's defence response against *Agrobacterium*, and it is possible to increase transient and stable transformation efficiencies by using SA inhibitors (PBZ and AIP).

To support the findings from **Chapter 2**, we verified the positive effect of SA inhibitors in enhancing gene transfer in *Lilium* (monocot) tepals via agroinfiltration (**Chapter 3**). Indeed, inclusion of PBZ or AIP at 10 µM concentration in MMA infiltration buffer did increase transient *GUS* expression tremendously in the tepals of *L. longiflorum* cv. 'Lincoln', up to the highest level, 70%-100%. Although PBZ was found to have a positive effect on the transformation efficiency, we could not detect any change in free SA levels (**Chapter 2**). Previous studies demonstrated that foliar spray of PBZ in *Lilium* 'Sorbonne' influenced changes in endogenous hormone contents (gibberellic acid (GA), abscisic acid (ABA), and indole-3-acetic acid (IAA)) (Zheng et al., 2012). These authors demonstrated that PBZ application could decrease GA but increase IAA which might promote carbohydrate and sucrose accumulation in lily bulbs for the formation of new scales. Azadi and coworkers (2010) reported that the use of sucrose in both full and modified MS medium showed a high frequency of transformation in *Lilium* x *formolongi* calli. Meanwhile, Ahn and Lee (2003) reported that various carbohydrates induced *GUS* expression in tobacco and soybean. These results indicated that enhancing transformation efficiency using SA inhibitors is not really straightforward, and happens perhaps through an interaction with other endogenous hormones. However, indications have been found that PBZ works as an effective method to enhance *Agrobacterium*-mediated transformation efficiency in both dicot and monocot plants.

As exposure to *Agrobacterium* represents a stress to the plant cells, it could lead to the formation of reactive oxygen species (Mullineaux and Baker, 2010). Adding an antioxidant such as lipoic acid (LA) helping plant cells to cope with this stress is reported to have a transformation enhancing effect (Dan et al., 2015; 2009). Addition of LA could serve as a way to improve transformation efficiency in *P. hybrida*, but not in *N. benthamiana* and *L. longiflorum* cultivar 'Lincoln'. The LA response varied between plant species, hence our study suggested that LA is a species-dependent enhancer rather than a general transformation enhancer as previously claimed by Dan et al. (2009).

Optimization of transient expression procedure

Production of stable transgenic plants with desired traits is the ultimate goal in genetic modification. However, production of stable transgenic plants often suffers from low transformation efficiencies and lengthy regeneration processes. The production of stable transgenic flowering plants requires several months. According to Dohm et al. (2002), the production of transgenic flowering rose with a maximum transformation efficiency of 3%

can be achieved within 9-12 months. For lily in our hands, it takes at least 8 months from start of transformation to rooted transgenic plantlets (Wang et al., 2012). Taking such a rooted transgenic lily plantlet, it will still take another 18 months before a flowr can be obtained. Although the highest transformation efficiency in lily that was claimed was 20% (Cáceres et al., 2011), they included a 1000 to 10.000-fold higher concentration of LA at 50 mM in their culture media during transformation compared to others (Dan et al., 2009; Dutt et al., 2011). Nevertheless, a simple method for monitoring transient gene expression is needed to test the functionality of the target genes without having to go through the entire genetic modification and plant regeneration process. Several protocols have been optimized for various plant species, most of them by means of particle bombardment (Benedito et al., 2005; Cohen et al., 2004; Wiltshire et al., 2017) and agroinfiltration (Carvalho et al., 2016; Pinthong et al., 2014; Yasmin and Debener, 2010; Zhao et al., 2017).

In **Chapter 3**, we developed and optimized a simple agroinfiltration method for *Lilium* tepals. The method used in this study was optimized based on previous agroinfiltration methods developed for rose (Yasmin and Debener, 2010), orchid (Pinthong et al., 2014), *N. benthamiana* (Suzuki et al., 2015) and *N. tabacum* (Yamagishi, 2016). Several factors that influence the expression efficiency after *Agrobacterium* infiltration were investigated. Among the most important factors identified thus far are the infiltration buffer, bacterial density, light conditions, time of co-cultivation and additives to enhance transformation efficiency.

Moreover, agroinfiltration as a non-destructive method is preferred over biolistic gene gun because the texture of *Lilium* tepals, that is slightly fleshy, can easily lead to browning and necrosis after being damaged. Ultimately we found that the gene transfer is optimal at the closed flower bud stage 4 (i.e. more than 10 cm in length, just before the flower opens) because the highest expression levels of anthocyanin-related genes in open flowers were observed when buds were infiltrated at this stage, as discovered in the Oriental hybrid lily cultivar 'Sorbonne' (Yamagishi, 2011), 'Perth' and 'Gran Tourismo' (**Chapter 4**). Then, we have to wait for 6 days until the flowers are open for harvest. The optimized method via syringe infiltration proved most suited for our experiments and could contribute to establishing an efficient stable transformation procedure in the future.

Molecular mechanisms regulating flower colour

Among pigments that give colour to plants, anthocyanins are the most extensively studied because they are not only responsible for plant colouration, but also beneficial for plant physiological processes and human health (Georgiev et al., 2014; Winkel-Shirley, 2001). The three major pigments of anthocyanins are cyanin (red to magenta), pelargonin

(orange to red) and delphinin (purple to blue) (Grotewold, 2006; Katsumoto et al., 2007; Tanaka and Ohmiya, 2008; Winkel-Shirley, 2001). The anthocyanin biosynthetic pathway is highly conserved amongst higher plant species and well-studied (Grotewold, 2006; Hichri et al., 2011).

The corresponding structural genes encoding enzymes involved in anthocyanin biosynthesis have been isolated from various plant species. In *Lilium*, anthocyanin structural genes such as phenylalanine ammonia-lyase, *PAL*; chalcone synthase, *CHS*; chalcone isomerase, *CHI*; flavanone 3-hydroxylase, *F3H*; flavonoid 3'-hydroxylase, *F3'H*; dihydroflavonol 4-reductase, *DFR*; and anthocyanidin synthase, *ANS* have been isolated, but the other genes such as glycosyltransferase, *GT*: acyltransferase, *AT* and methyltransferase, *MT* have not (Lai et al., 2012; Liu et al., 2011; Nakatsuka et al., 2003; Suzuki et al., 2016; 2015). The glycosylation, acylation and methylation of anthocyanidins causes the colour to become slightly redder/bluer and stable (Tanaka et al., 2010). **Chapter 4** describes that high transcript accumulation of eight structural genes (*CHSa, CHSb, CHIa, CHIb, F3H, F3'H, DFR* and *ANS*) was observed in the coloured tepals of 'Gran Tourismo' (red tepals), as well as in 'Perth' (pink tepals), but to a lesser extent. The white tepals of 'Rialto' and 'Lincoln' showed the lowest transcript accumulation. As previously reported in other plants, the expression of anthocyanin biosynthesis-related genes in the flowers are under developmental control (Koes et al., 2005; Nakatsuka et al., 2003). During flower development of 'Gran Tourismo', no or low anthocyanin structural gene expression was detected at the early bud stage 1 and early bud stage 2, respectively. The expression increased at stage 3 buds, peaked at stage 4 buds, and slightly dropped at stage 5 open flowers.

Many *Lilium* species show anthocyanin pigmentation of the whole tepals, and this is assumed to be regulated by an interaction between *MYB12* and *bHLH2* transcription factors (Nakatsuka et al., 2009; Yamagishi, 2011,2016; Yamagishi et al., 2012). In 'Gran Tourismo' and 'Perth' tepals, the expression profiles of structural genes are correlated with the expression patterns of *MYB12* and *MYB15*, suggesting some co-regulation. The highest expression of *MYB12* is seen in the red tepals of 'Gran Tourismo', followed by the pink tepals of 'Perth', and this result is in parallel with their anthocyanin contents. Moreover, expressing 'Gran Tourismo' *MYB12* into 'Perth' tepals resulted in the deepest pink colouration at the infiltrated area compared to that of 'Perth' and 'Rialto' *MYB12*. Based on these results, it is suggested that *MYB12* is a factor that influence the amounts of anthocyanin in 'Gran Tourismo' and 'Perth'. The importance of *MYB12* in anthocyanin accumulation has been reported in several Oriental and Asiatic hybrid lilies (Lai et al., 2012; Yamagishi and Nakatsuka, 2017; Yamagishi, 2011; Yamagishi et al., 2014, 2012), however, *MYB15* has been

recently discovered from *Lilium regale*, controlling anthocyanin pigmentation in the flower buds, leaves and bracts (Yamagishi 2016). *MYB15* is also transcribed in 'Gran Tourismo' and 'Perth' tepals, which are distantly related to *L. regale*. However, the Asiatic hybrid lily cultivar 'Grand Cru' which has bright yellow tepals with red spots, does not contain *MYB12*, but instead, contains *MYB15*-like, *MYB16, MYB17* and *MYB18* transcription factors (Yamagishi, 2018), which might indicate that *MYB15* is not only involved in regulating the transcription of structural genes of the anthocyanin biosynthetic pathway, but perhaps also of (some genes of) the carotenoid biosynthetic pathway. Further research on this can be quite exciting.

Towards understanding the genetic background behind white tepal colour

Lilium contains cyanidin 3-O-β-rutinoside as the major anthocyanin and cyanidin 3-O-β-rutinoside-7-O-β-glucoside as a minor anthocyanin in their coloured tepals (Nørbæk and Kondo 1999). With a single anthocyanidin, namely cyanidin, accumulating in 'Gran Tourismo' and 'Perth' tepals, no measurable levels of dihydroquercetin (DHQ, the substrate for DFR leading to leucocyanidin) but a high accumulation of dihydrokaempferol (DHK) was observed in all four lily cultivars. This result indicated the presence, activity and functionality of early structural genes (*CHSa, CHSb, CHIa, CHIb, F3H*) in the pathway in all cultivars, although the expression of *CHSa* and *CHSb* seemed to be very weak in 'Rialto' and *CHSb* was very weak in 'Lincoln'. Based on the results obtained, we postulate that the suppression of the anthocyanin biosynthetic pathway in flowers would be after the step where DHK was produced (as shown in Fig. 2). This further indicated that the missing enzyme could be from F3'H or a later gene in the biosynthetic pathway, which are mainly regulated by the *MYB12* transcription factor (Yamagishi, 2011; Yamagishi et al., 2010).

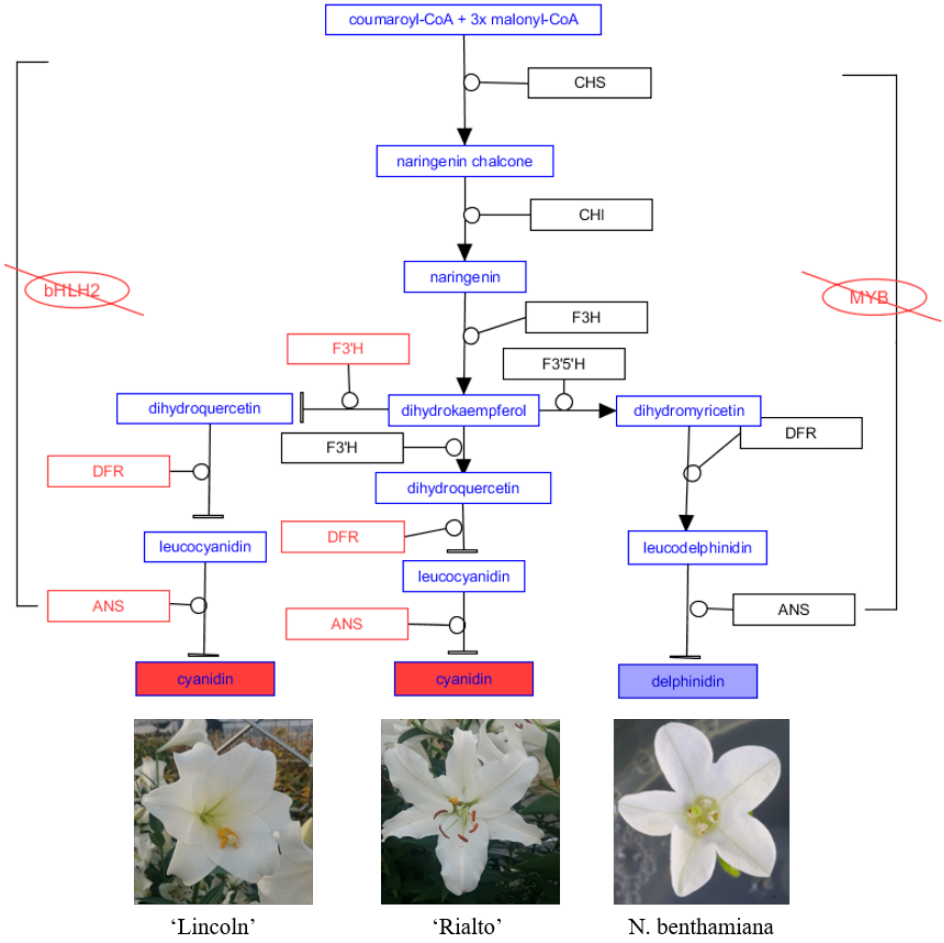

Fig. 2. A simplified scheme of anthocyanin biosynthetic pathway in *L. longiflorum* cultivar 'Lincoln', the Oriental hybrid lily cultivar 'Rialto' and *N. benthamiana* flowers. The inhibition of anthocyanin biosynthesis is indicated by the red boxes or spheres for the three white-flowered genotypes.

There are several mechanisms including mutations, absence of genes, and differential expression levels of various genes that generate white flowers in lilies, and the mechanisms mentioned above are variable depending on the plant species being considered. In gentian (*Gentiana triflora*), there are two white-flowered cultivars; one is likely caused by deficiency in *ANS* transcription and the other one is presumably because of a mutation in one of the regulatory genes (Nakatsuka et al., 2005). In Asiatic hybrid lily cultivars 'Navona' and

'Silver Stone', insufficient expression of *MYB12,* which regulates anthocyanin pigmentation causes the white tepal colour (Yamagishi et al., 2012). In the Oriental hybrid lily cultivar 'Casa Blanca' and its putative parent *Lilium auratum* var. *platyphyllum,* no expression of *MYB12* is likely the cause of white tepal colour (Yamagishi et al., 2014). In the other Oriental hybrid lily cultivar 'Rialto' and *Lilium speciosum* (white tepal and red anther line), the white tepal colour is likely caused by a W-to-L amino acid substitution in the R2-repeat of *MYB12*, which is expressed in tepals but unable to activate the transcription of structural genes (Suzuki et al., 2015; Yamagishi, 2011; Yamagishi et al., 2012).

In our study, the W-to-L substitution was also found in the *MYB12* of the pink cultivar 'Perth' (**Chapter 4**). Analysis of the transcription of *MYB12* from 'Gran Tourismo' (red tepals), 'Perth' (pink tepals), and 'Rialto' (white tepals) after agroinfiltration in 'Perth' tepals showed that all the isolated *MYB12* genes were able to activate *DFR* and *ANS*, resulting in increased anthocyanin contents (**Chapter 4**). These observations question the suggested effect of the W-to-L amino acid substitution in the R2 repeat, which would supposedly completely suppress anthocyanin biosynthesis (Suzuki et al., 2015). On top of the mutated *MYB12*, we also found in 'Rialto' that *DFR* was not transcribed in tepals, which is the likely cause for the white colouration. A nonsense mutation which led to a premature stop codon in *DFR*-wy supposedly causes the white tepal and yellow anther phenotype in *L. speciosum* (Suzuki et al., 2015). In **Chapter 4**, we demonstrated that partial protein sequence of 'Rialto' *DFR* was unique compared to other Oriental lilies and *L. speciosum* by four unique amino acid substitutions. However, no critical mutations such as a premature stop codon that could impair DFR protein function as reported by Suzuki and his coworkers (2015) was found. Moreover, the active site and the protein pocket were detected in all three *DFR* copies checked from 'Gran Tourismo', 'Perth' and 'Rialto'. One possible explanation for this untranscribed *DFR* from 'Rialto' is variation at the *DFR* upstream sequence part, whereby the transcription factors might not be able to bind to it. In parallel with this, we were unable to PCR-amplify and sequence the beginning of *DFR* from 'Rialto' using the same primer pairs used to sequence the full length of the gene from 'Perth' and 'Gran Tourismo'.

The absence of two structural genes *F3'H*, *DFR* and one transcription factor *basic helix-loop-helix 2* (*bHLH2*) gene are likely the causes of white colour in *L. longiflorum* cv. 'Lincoln' tepals (**Chapter 4**). We were unable to amplify genomic sequences of two structural genes (*F3'H* and *DFR*) and one transcription factor (*bHLH2*), possibly explaining the absence of transcripts of the genes as detected by qPCR. However, anthocyanins seem to be formed in the stems (**Chapter 4**), indicating the presence and expression of *F3'H* and *DFR* genes there. One possible explanation for this is variation in the nucleotide sequences between *L. longiflorum* and other *Lilium* spp. Gene specific primer pairs (used for gDNA

PCR-amplification or qPCR), which successfully amplified *F3'H, DFR* and *bHLH2* genes in all three Oriental cultivars 'Gran Tourismo' 'Perth' and 'Rialto', did not work in 'Lincoln'. The primers were designed based on specific gene sequences from the Oriental and Asiatic hybrid lilies. The lack of gene sequences information in *L. longiflorum* available in databases is the main limiting factor in this study. Most of the transcriptomic studies were performed in the Asiatic and Oriental hybrid lilies (Moreno-Pachon et al., 2016; Shahin et al., 2012; Suzuki et al., 2016). Therefore, further RNA-seq analysis for anthocyanin-related genes expression in *L. longiflorum* is necessary in order to determine the main transcriptomic differences between 'Lincoln' and 'Rialto'.

Genetic modification of flower colour

Manipulation of the transcription factors is considered an ideal strategy to alter the expression of multiple structural genes, thus influencing anthocyanin intensity. In **Chapter 3,** we successfully transformed *N. benthamiana* flowers and increased the colour intensity in the Oriental hybrid lily cultivar 'Perth' (pink tepals) by introducing a dicot *Anthirrhinum majus ROS1* (a *MYB*-like) and a *DEL* (a *bHLH*-like) transcription factor separately or combined. Introduction of *ROS1* alone is sufficient to induce the highest transcription levels of anthocyanin structural genes (*NbCHS, NbF3H, NbDFR* and *NbANS*) in *N. benthamiana.* It is likely that the endogenous *MYB* transcription factor is not functional (Fig. 2), and therefore *ROS1* complements through an interaction with an endogenous *DEL*-like transcription factor present in *N. benthamiana*. In 'Perth', the combination of *ROS1+DEL* stimulates the highest transcription of early (*LhCHSa, LhCHSb, LhCHIa, LhCHIb, LhF3'H)* and late structural genes (*LhDFR* and *LhANS*), indicating the importance of both *MYB*- and *bHLH*-type transcription factors being present in lily.

The transcription factor genes expressed under control of the *Floral Binding Protein 1* (*FBP1*) promoter, a flower-specific promoter from *Petunia hybrida* (Angenent et al., 1993), were found to stimulate colour formation only in the flowers, in both *N. benthamiana* and lily 'Perth'. This indicated that *FBP1* is a suitable promoter for driving tissue-specific expression until the late flowering stage, particularly in flowers of both dicots and monocots. In contrast, the use of the *35S* promoter produced too much anthocyanin in all *N. benthamiana* plant parts, which may have interfered with plant development, and finally resulted in a stunted plant, suggesting that the *35S* promoter is not suitable for modification of flower colour.

Recently, Wei and coworkers (2017) reported that anthocyanin biosynthesis was induced in the transformed plantlets of Oriental hybrid lily cultivar 'Sorbonne' after the introduction of *ROS1+DEL* under control of the *35S* promoter. The colour however was not stable and had faded back to green after the plantlets were rooted. In the green tissue

like leaves, anthocyanin accumulation might have been masked by chlorophyll and variation in vacuolar pH during leaf development might cause the loss of anthocyanin contents (Wei et al., 2017). However in flower tissue, other *MYB*-like transcription factors such as *MYB12* and *MYB15* have been shown to induce anthocyanin accumulation in the Asiatic and Oriental hybrid lily tepals (Yamagishi and Nakatsuka, 2017; Yamagishi, 2016; 2011). It was speculated whether the change of flower colour in transformed lily will remain stable, as demonstrated in the flowers of transformed *N. benthamiana* (**Chapter 3**). Future research with flower colour in lily and in other monocots can result in the isolation and expression of other structural genes and regulatory genes of the anthocyanin biosynthetic pathway under control of *FBP1* flower-specific promoter.

In **Chapter 5**, we investigated the hypothesized causes for white flower colour in lily. The functionality of the *F3'H* gene from *L. speciosum* and of the *DFR, ANS and MYB12* genes from 'Gran Tourismo' were determined by complementation analysis in *Arabidopsis* mutants and/or transient expression in Lily cv. 'Perth' tepals. On top of that, the genes were transiently expressed with or without snapdragon *ROS1* and *DEL* transcription factor genes in 'Perth', 'Rialto' and 'Lincoln' tepals. This study demonstrated that the isolated copies of *F3'H* and *ANS* from lily were functional genes that proved able to induce anthocyanin biosynthesis in the Oriental hybrid lily cv. 'Perth' and restored the gene function in *Arabidopsis* mutants.

Introducing *DFR* from 'Gran Tourismo' in 'Perth' tepals seemed to work in the beginning, as anthocyanin accumulated in the infiltrated tepals. However, sequence checking on the increased *DFR* expression levels in 'Perth' tepals showed that the transcribed gene was in fact the resident 'Perth' *DFR* gene. In general, stimulation of expression of genes after introduction of a non-functional gene copy could be that of oxidative stress caused by *Agrobacterium* (Wang et al., 2013). On the other hand, we demonstrated that the endogenous copy of *DFR* in 'Perth' is easily stimulated to high expression levels by the introduction of multiple genes. Perhaps even very low levels of expression of a faulty constructed *DFR* copy or of an isolated low-expressed gene copy of *DFR* from 'Gran Tourismo' could already trigger resident *DFR* gene expression in 'Perth'. Testing complementation of an *Arabidopsis DFR* mutant showed that the *DFR* gene copy isolated from 'Gran Tourismo' was not active and express hence anthocyanin production could not be restored, although we did first sequence the *DFR* construct produced in *E. coli* as a control. Looking at the prominent red colour of 'Gran Tourismo' tepals, it should contain one functional copy of *DFR* which is able to convert dihydroquercetin to leucocyanidin.

MYB12 from 'Gran Tourismo' induced anthocyanin accumulation in 'Perth' tepals through activation of *DFR* and *ANS*. However, co-expression of multiple genes failed to induce anthocyanin biosynthesis in the white-flowered 'Rialto' and 'Lincoln'. The expression

levels of *F3'H, ANS* and *MYB12* could be upregulated, but not expression of *DFR*. This prompted us to test more *DFR* constructs, e.g. from *Gerbera* (*DFR1, DFR2* and *DFR3*) and *Petunia* (*DFRa*) that have been proven to be functional in other systems, but also without success so far in lily. The absence of a functional and suitable *DFR* gene copy seemed to be the main bottleneck in the pathways of 'Rialto' and 'Lincoln' and could unfortunately not be compensated by the *DFR* copy from 'Gran Tourismo' that we isolated and which turned out to be non-functional. Nevertheless, a deeper RNA sequencing identifying a mutation in the *DFR* gene copies of 'Rialto' and 'Lincoln' is needed to confirm the hypothesis.

Concluding remarks and future perspective

In this study, we have been focussing on modification of flower colour in *Lilium* spp. via genetic engineering. However, *Lilium* transformation is a difficult and a lengthy process. Although *Agrobacterium*-mediated and direct gene transfer have been successfully used in *Lilium* transformation, the transformation efficiency is still low and varies between genotypes. In *Agrobacterium*-mediated transformation, two partners are involved, the plant and the *Agrobacterium.* Success in *Agrobacterium*-mediated transformation is greatly influenced by a balanced interaction between the two partners. Indications have been found that salicylic acid (SA) was responsible for raising the plant's defence response against *Agrobacterium*, and it was possible to increase transformation efficiencies by using SA inhibitors (paclobutrazol, PBZ and 2-aminoindan-2-phosphonic acid, AIP). 10 µM PBZ was selected as the optimal concentration that enabled enhancing transient and stable transformation efficiencies in *P. hybrida*; it was subsequently found that it also enhanced transient *GUS* expression in *Lilium* tepals by 70-100%.

Anthocyanin biosynthesis in plants is correlated with the transcription of anthocyanin structural genes, which are mainly regulated by a *MYB*-type and *bHLH*-type transcription factor. Snapdragon *ROS1* (a *MYB*-type) and *DEL* (a *bHLH*-type) transcription factors were found to activate anthocyanin biosynthesis in the flowers of *N. benthamiana* and the Oriental hybrid lily cultivar 'Perth'. We also established the suitability of *FBP1*, a flower-specific promoter from petunia, in flower colour modification in both *N. benthamiana* and the Oriental hybrid lily cultivar 'Perth'. However, introducing *ROS1* and *DEL* into the white flowered *L. longiflorum* cultivar 'Lincoln' and the Oriental hybrid lily cultivar 'Rialto' could not activate anthocyanin pigmentation in both cultivars, suggesting more deficiencies in the pathway. We performed gene expression analysis during multiple flower developmental stages and gene-specific amplification on cDNA and gDNA to investigate the genetic background behind the white colouration in 'Rialto' and 'Lincoln' as well as to investigate mechanisms regulating flower colour in the coloured Oriental cultivars 'Perth' and 'Gran Tourismo'. The absence of

two structural genes, (*F3'H* and *DFR*) and one transcription factor (*bHLH2*) was likely the cause for white colouration in 'Lincoln'. In 'Rialto', *DFR* was present, but not expressed. Together with that, insufficient expression of *ANS* was detected in 'Lincoln' and 'Rialto'.

To test the hypothesized causes of white colouration in 'Lincoln' and 'Rialto, we characterized the functionality of *F3'H* from *L. speciosum*, and *DFR, ANS* and *MYB12* from 'Gran Tourismo' by complementation analysis in *Arabidopsis* mutants and/or transient expression in 'Perth' tepals. The introduced *F3'H, ANS* and *MYB12* from lily were functional genes, but *DFR* was not. Co-expression of the genes with or without *ROS1* and *DEL* in the white flowered 'Lincoln' and 'Rialto' failed to induce anthocyanin biosynthesis in both cultivars. The expression levels of *F3'H, ANS* and *MYB12* were upregulated, but hardly any expression of *DFR* was detected. As a result, *DFR* seems to be the main bottleneck for not having colour in 'Lincoln' and 'Rialto'. A follow up of this research should focus on a careful selection and characterization of a functional and suitable *DFR* gene copy from other lilies or another crop in order to produce novel flower colour in both cultivars.

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Summary

Lily (genus *Lilium,* family Liliaceae) is one of the most important ornamental crops for the production of flower bulbs and cut flowers. As cut flowers, lily is ranked at number four in the Dutch list of economically important ornamental crops after rose, chrysanthemum and tulip (Flora Holland, 2016). Lily is taxonomically classified into sections including *Archelirion, Sinomartagon* and *Leucolirion.* Interspecific hybridization is a much used method in lily breeding. Oriental hybrids (O) are the result of interspecific crosses between *Lilium* species from the section *Archelirion.* Asiatic hybrids (A) are derived from interspecific crosses between *Lilium* species belonging to the section *Sinomartagon,* whilst Longiflorum hybrids (L) are derived from interspecific crosses between species belonging to the section *Leucolirion.* The Asiatic and Oriental hybrids occupy a prominent place in the world flower market, and the variation in flower colour is their key attractive factor. The Asiatic hybrids display a huge variation in flower colours ranging from yellow, orange, pink, red and white. The Oriental hybrids have big, showy flowers in pink, red and white. On the other hand, Longiflorum hybrids only have white flowers, and thus varying their flower colours would be of potential commercial value. Wide inter-sectional breeding has been attempted to produce LA or LO hybrids with altered flower colours, this however resulted in F1 hybrids that are highly sterile. Hence, conventional breeding might be inefficient for introducing variation in flower colours in *L. longiflorum* due to hybrid sterility and deficiency of genetic resources in related, crossable species.

An alternative is provided by the technology of genetic modification, but lily is among the monocot species that is recalcitrant to genetic transformation by *A. tumefaciens* and thus exhibits a low transformation efficiency (TE). There is a need to enhance TE in lily. To enhance *Agrobacterium*-mediated TE, the effects of adding salicylic acid (SA) and SA inhibitors (paclobutrazol, PBZ and 2-amidoindane-2-phosphonic acid, AIP) in the culture media during transformation of *Petunia hybrida* and *Nicotiana benthamiana* were evaluated (**Chapter 2**). Our results indicated that SA is responsible for the plant's defence response against *Agrobacterium* and blocking SA biosynthesis using SA inhibitors could promote TE. Another way to enhance TE is to improve the plant's condition to cope with oxidative stresses caused by *Agrobacterium.* The use of lipoic acid as an antioxidant would help plant cells to cope with such stresses thereby improving TE. Adding lipoic acid at 10 µM enhanced gene transfer efficiency in *P. hybrida,* but not in *N. benthamiana*. Since the response to lipoic acid varies between plant species, it is suggested that lipoic acid is a species-dependent enhancer. Nevertheless, indications have been found that SA inhibitors and lipoic acid could promote transformation efficiency in dicot model plants and possibly in monocots too.

In **Chapter 3**, we have validated the role of SA inhibitors in lily transformation by agroinfiltration. The use of SA inhibitors at 10 µM increased *GUS* transient expression tremendously in *L. longiflorum* cultivar 'Lincoln' tepals. Because lily transformation is proven to be difficult and time consuming, we developed and optimized a simple agroinfiltration method in order to monitor transient gene expression of anthocyanin structural genes and regulatory genes in lily tepals. The *ROSEA1* (*ROS1,* a *MYB*-type) transcription factor (TF) from *Anthirrhinum majus* which resembles *Lilium MYB12* in the sequence profile interacts with *DELILA* (*DEL*, a *bHLH*-type TF) to activate the production of delphinidin in *N. benthamiana* and cyanidin in the Oriental hybrid lily cultivar 'Perth'. Unfortunately, the introduction of *ROS1+DEL* did not bring about any phenotypic changes to the white flowered 'Rialto' and 'Lincoln', suggesting that some structural gene(s) involved in the anthocyanin biosynthetic pathway in these two cultivars is/are lacking or mutated. The expression of *ROS1* and *DEL* under control of the flower-specific promoter, *Floral Binding Protein 1* (*FBP1*) from *Petunia hybrida* appeared to be important to achieve significant anthocyanin production in the flowers, in both *N. benthamiana* and lily 'Perth'. In contrast, the use of the *35S* promoter produced too much anthocyanin in all *N. benthamiana* plant parts and finally resulted in a stunted plant. These results indicated that the *35S* promoter often produces undesirable results, hence a tissue-specific promoter is needed in flower molecular breeding.

In **Chapter 4**, we determined molecular mechanisms regulating flower colour in lilies by an expression analysis of anthocyanin biosynthesis-related genes during multiple flower developmental stages. The presence 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. The research described in this chapter was carried out with two coloured cultivars, the Oriental hybrid lily cultivars 'Perth' (pink tepals) and 'Gran Tourismo' (red tepals) and two white-flowered cultivars, 'Lincoln' and 'Rialto'. In the coloured cultivars, the expression of eight anthocyanin structural genes (*CHSa, CHSb, CHIa, CHIb, F3H, F3'H, DFR* and *ANS*) is under developmental control and is associated with the accumulation of cyanidin. The transcription of anthocyanin structural genes is regulated by *MYBs* and *basic helix-loop-helix* 2 (*bHLH2*) transcription factors. The *MYB12* and *MYB15* play an important role in regulating the anthocyanin accumulation, but *MYB12*, still, is the main factor that influences anthocyanin intensity. Different molecular mechanisms that generate white tepal colour in 'Lincoln' and 'Rialto' have been suggested by us. The absence of two structural genes *F3'H*, *DFR* and one transcription factor *basic helix-loop-helix 2* (*bHLH2*) gene resulting in no transcript levels of the genes detectable by qPCR was likely responsible for the white tepal colour in 'Lincoln'. It was mentioned before that 'Rialto'

is white because of a mutation in *MYB12*. We found that *DFR* was present in 'Rialto', but not expressed in flowers. On top of that, insufficient expression of *ANS* might also have contributed to the white tepal colour in 'Lincoln' and 'Rialto'.

To investigate the hypothesized causes for white tepal colour in lily, the functionality of a synthetic *F3'H* from *Lilium speciosum,* and of isolated *DFR, ANS* and *MYB12* genes from 'Gran Tourismo' were determined by complementation analysis in *Arabidopsis* mutants and/or transient expression in lily 'Perth' tepals (**Chapter 5**). The *F3'H* and *ANS* from lily were two functional genes that proved able to induce anthocyanin biosynthesis in 'Perth' and restored the gene function in *Arabidopsis* mutants. Testing complementation of an *Arabidopsis* mutant and sequencing the increased *DFR* levels in 'Perth' introduced by *DFR* from 'Gran Tourismo' showed that the introduced gene copy was not functional. *MYB12* from 'Gran Tourismo' induced anthocyanin accumulation in 'Perth' tepals through activation of *DFR* and *ANS*. However, co-expression of multiple genes with or without *ROS1* and *DEL* failed to induce anthocyanin biosynthesis in the white-flowered 'Rialto' and 'Lincoln' due to the complexity of the anthocyanin biosynthetic pathway. The absence of a functional and suitable *DFR* gene copy seemed to be the main bottleneck in the pathway of 'Rialto' and 'Lincoln'. Thus, introducing a functional and suitable *DFR* gene copy from lily or another ornamental crop remains to be investigated in order to produce novel flower colour in both cultivars. The information gained in the research described in this thesis should aid in future efforts in designing rational strategies for the modification of flower colours in lilies.

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Nur Fatihah Hasan Nudin

Wageningen, 30 November 2018.

Curriculum Vitae

Nur Fatihah Hasan Nudin was born in 3rd July 1984 in Kelantan, Malaysia. In 2006, she accomplished her bachelor degree in Biology from Universiti Putra Malaysia. Then in 2007, she was appointed as a science officer at Universiti Sultan Zainal Abidin (UniSZA). She continued with her Master degree in Plant Genetic Resources at the University of Birmingham, UK. After graduation in 2009, she was promoted to lecturer at the School of Agriculture and Biotechnology, Faculty of Bioresources and Food Industry, UniSZA. In September 2013, she received funding from the Ministry of Higher Education of Malaysia (MOHE) to continue her PhD study under supervision of Dr Frans Krens and Prof. Dr Richard Visser at the Plant Breeding, Wageningen University and Research, The Netherlands. This thesis presents the outcomes of her five years PhD research on "Molecular determination and genetic modification of flower colour in *Lilium* spp.". Nur Fatihah Hasan Nudin will continue her research and teaching career as lecturer at UniSZA, which is located in Terengganu, Malaysia. She can be reached by email: fatihah@unisza.edu.my.

The following is a list of her publications in refereed journals:

Hasan Nudin, N. F., van Kronenburg, B., Tinnenbroek, I., Krens, F. A. (2015). The importance of salicylic acid and an improved plant condition in determining success in Agrobacterium-mediated transformation. Acta Horticulturae, 1087, 65–69. https://doi.org/10.17660/ActaHortic.2015.1087.7

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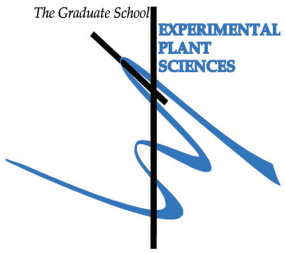
Education Statement of the Graduate School

Experimental Plant Sciences



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Date: 30 November 2018
Group: Laboratory of Plant Breeding
University: Wageningen University & Research

1) Start-Up Phase	*date*
► **First presentation of your project**	
Genetic modification of flower colour in ornamental crops	22 Sep 2014
► **Writing or rewriting a project proposal**	
Genetic modification of flower colour in ornamental crops	Mar 2014
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 **

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student days 'Get2Gether', Soest, NL	29-30 Jan 2015
EPS PhD student days 'Get2Gether', Soest, NL	28-29 Jan 2016
EPS PhD student days 'Get2Gether', Soest, NL	09-10 Feb 2017
► **EPS theme symposia**	
EPS Theme 3 Symposium 'Metabolism and Adaptation', Utrecht, NL	10 Feb 2015
EPS Theme 3 Symposium 'Metabolism and Adaptation', Amsterdam, NL	23 Feb 2016
EPS Theme 4 Symposium 'Genome Biology', Wageningen, NL	16 Dec 2016
EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents' & Willie Commelin Scholten Day, Wageningen, NL	23 Jan 2017
EPS Theme 3 Symposium 'Metabolism and Adaptation', Wageningen, NL	14 Mar 2017
► **National meetings (e.g. Lunteren days) and other National Platforms**	
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	14-15 Apr 2014
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	13-14 Apr 2015
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	11-12 Apr 2016
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	10-11 Apr 2017

►	**Seminars (series), workshops and symposia**	
	Seminar: Prof.dr. George Coupland - 'Seasonal flowering in annual and perennial plants'	19 Jan 2015
	Seminar: Dr. Sotirios Fragkostefanakis - 'Alternative splicing of a heat stress transcription factor mediates thermotolerance in tomato'	02 Nov 2016
	Symposium: Plant Research Day (Plant Breeding), Wageningen, NL	30 Sep 2014
	Symposium: 'All inclusive Breeding: Integrating high-throughput science', Wageningen, NL	16 Oct 2014
	Symposium: 2nd Wageningen PhD symposium 'Connecting Ideas, Combining Force', Wageningen, NL	06 May 2015
	Symposium: 'WURomics: Technology-Driven Innovation for Plant Breeding', Wageningen, NL	15 Dec 2016
	Symposium: Wageningen PhD symposium 'Science: From local to global', Wageningen, NL	03 May 2017
	Symposium: NVPW Symposium, Wageningen, NL	09 Jun 2017
►	**Seminar plus**	
►	**International symposia and congresses**	
	EUCARPIA 2015, Melle, Belgium	28 Jun - 02 Jul 2015
	Plant Omics and Biotechnology for Human Health, Gent, Belgium	21-24 Nov 2016
	5th Plant Genomics & Gene Editing Congress, Amsterdam, NL	16-17 Mar 2017
►	**Presentations**	
	Poster: The importance of salicylic acid and an improved plant condition in determining success in Agrobacterium-mediated transformation, EUCARPIA 2015, Melle, Belgium	28 Jun-02 Jul 2015
	Poster: Expression of anthocyanin biosynthesis-related genes and metabolite profiles in Lilium cultivars during flower development, Plant Omics and Biotechnology for Human Health, Gent, Belgium	21-24 Nov 2016
	Poster: The ROSEA1 and DELILA transcription factors control anthocyanin accumulation in N. benthamiana and Oriental lily flowers, 5th Plant Genomics & Gene Editing Congress, Amsterdam, NL	16-17 Mar 2017
	Poster: Anthocyanin biosynthesis in N. benthamiana and Oriental hybrid lily cv. 'Perth', Annual Meeting 'Experimental Plant Sciences', Lunteren, NL	10-11 Apr 2017
	Talk: Molecular mechanisms regulating flower colour in Lilium cultivars, EPS PhD Studentd Days 'Get2Gether', Soest, NL	09-10 Feb 2017
	Talk: Control of anthocyanin biosynthesis by MYB- and bHLH-type transcription factors, NVPW Symposium, Wageningen, NL	09 Jun 2017
►	**IAB interview**	
►	**Excursions**	
	Visit to the company Enza Zaden, Enkhuizen, NL	12 Jun 2015
	Subtotal Scientific Exposure	*17.3 **

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Advanced course 'Transcripton Factors and Transcriptional Regulation', Wageningen, NL	17-19 Dec 2013
Advanced course 'Bioinformatics – A User's Approach', Wageningen, NL	25-29 Aug 2014
Advanced course 'The power of RNA-Seq', Wageningen, NL	10-12 Feb 2016
Course, 'Basic Statistics', Wageningen, NL	17-24 May 2016
Advanced course 'Microscopy and Spectroscopy in Food and Plant Science, Wageningen, NL	15-17 May 2017
► **Journal club**	
Participation in literature discussion group at Plant Breeding, Wageningen UR	
► **Individual research training**	
Subtotal In-Depth Studies	*5.8 **

4) Personal Development	*date*
► **Skill training courses**	
Competence Assessment, Wageningen, NL	02 Jun 2015
Efficient Writing Strategies, Wageningen, NL	Apr-May 2016
The Essentials of Scientific Writing and Presenting, Wageningen, NL	02-12 Dec 2016
Information Literacy including EndNote Introduction, Wageningen, NL	06-07 Dec 2016
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.4 **

TOTAL NUMBER OF CREDIT POINTS	***34.0 ****
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits.	

** A credit represents a normative study load of 28 hours of study.*

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