AN INVESTIGATION OF THE ROLES OF MYB TRANSCRIPTION FACTORS IN ORCHID *DENDROBIUM HYBRIDA* FLOWER DEVELOPMENT

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ABSTRACT

MYB genes encode a large family of transcription regulators in eukaryotes. Most plant *MYB* genes belong to the R2R3-MYB subfamily. MYB gene family members have been studied in various plant species and play roles in the regulation of plant development, hormone signalling, defence response and secondary metabolism. Orchid plants play an important role in the floral industry owing to their unique flower shapes, fragrance, colours and keeping quality. Flower colours are influenced by flower pigmentation, vacuolar compounds, pH, metal ions and shape of epidermal cells. *R2R3-MYB* genes regulate flower pigmentation, flower shape and cell shape therefore have importance in the development of new varieties of orchids. Polymerase chain reaction with sequence consensus based primer was used to amplify thirty-seven partial cDNA fragments representing *R2R3-MYB* gene sequences were isolated from *Dendrobium hybrida*. Phylogenetic analysis with IT3F: An Interspecies Transcription Factor Function Finder for Plants (http://jicbio.bbsrc.ac.uk/IT3F) of the translated *R2R3-MYB* gene sequences revealed that *MYB* sequences from *Dendrobium hybrida* are clustered into six subgroups. Nine of the translated *DhMYB* genes were clustered in subgroup 9 which are known to be involved in cell shape development. Production of dsRNA, sense and antisense RNAs were synthesized in vitro from the corresponding DNA plasmids by using double T7 RNA polymerase. RNAi knock down of the expression of *DhMYB* using dsRNA approach showed significant reduction of *DhMYB* gene expression compared with untreated control. Cell shape scanning electron microscopy of adaxial epidermal cells within domain 1 of the lip of flowers treated with *DhMYB* dsRNA had flattened epidermal cells whilst those of control flowers were conical – papillate. We suggest that *DhMYB* cDNA controls cell morphogenesis in the lip epidermis.

ABSTRAK

Gen MYB mengekod keluarga besar pengawal transkripsi dalam eukaryotes. Kebanyakan gen MYB tumbuh-tumbuhan tergolong dalam subfamili R2R3-MYB. Ahli keluarga gen MYB telah dikaji dalam pelbagai spesis tumbuh-tumbuhan dan memainkan yang peranan dalam kawalan perkembangan tumbuh-tumbuhan, isyarat hormon, tindak balas pertahanan dan metabolisme sekunder. Orkid memainkan peranan yang penting dalam industri bunga kerana bentuk bunganya yang unik, wangian, warna dan kualiti. Warna-warna bunga orkid adalah dipengaruhi oleh pigmentasi bunga, sebatian vacuolar, pH, ion logam dan juga bentuk sel-sel epidermis. R2R3-MYB gen mengawal pempigmenan bunga, bentuk bunga dan bentuk sel. Oleh itu, ia mempunyai kepentingan dalam pembentukan varieti baharu orkid. Tindak balas rantai polimerase dengan urutan berasaskan konsensus telah digunakan untuk menjelaskan bahawa tiga puluh tujuh serpihan cDNA separa mewakili jujukan gen R2R3-MYB telah diekstrakkan daripada *Dendrobium hybrida*. Analisis filogenetik gen dengan IT3F: Satu Pencari Fungsi Faktor Transkripsi Interspecies untuk Tumbuhan (http://jicbio.bbsrc.ac.uk/IT3F) menunjukkan bahawa jujukan R2R3-MYB dari *Dendrobium hybrida* dapat dikategorikan kepada enam subkumpulan. Sembilan gen DhMYB berkelompok dalam kumpulan kesembilan yang berfungsi dalam perkembangan bentuk sel. Hasil daripada dsRNA, deria dan antideria RNAs adalah disintesis dalam vitro dari plasmids DNA yang sepadan dengan menggunakan polimerase T7 RNA berganda. Proses RNAi atas transkripsi *DhMYB* dengan menggunakan pendekatan dsRNA menunjukkan pengurangan transkripsi gen *DhMYB* secara ketara sekiranya dibandingkan dengan kawalan pokok orkid yang tidak dirawat. Mikroskopi elektron penskanan bentuk sel-sel epidermis adaksial dalam domain 1 bibir bunga yang dirawat dengan DhMYB dsRNA telah diratakan, manakala bentuk sel-sel epidermis adaksial dalam domain 1 bibir bunga kawalan adalah berbentuk kon - papilat.

Kami mencadangkan bahawa DhMYB cDNA mengawal sel morfogenesis dalam epidermis bibir bunga.

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List of Abbreviations

F	forward
R	reverse
ml	mililitre
CTAB	Cetyl _trimethylammonium bromide
EDTA	Ethylenediaminetetraacetic acid
PVP	Polyvinylpyrrolidone
NaCl	Sodium chloride
µL	Microlitre
Cl	Chlorine
g	Gravity
v/v	Volume to volume
LiCl	Lithium chloride
NaOAc	sodium acetate
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
RACE	Rapid amplification of cDNA ends
DEPC	Diethylpyrocarbonate
PCR	Polymerase chain reaction
CDNA	Complementary DNA
OD	optical density

RPM	Revolutions per minute
MW	Molecular weight
mM	Milimolar
MgSO4	Magnesium sulfate
MGCL2	Magnesium chloride
LB	Luria Bertani
AMP	Amplicillin
Bp	Base pair
CaCl2	Calcium chloride
dNTP	Deoxynucleotide triphosphate
HCI	Hydrochloric acid
IPTG	Isopropyl-beta-D-thiogalactopyranoside

Chapter 1.0 Introduction

Orchidaceae is distinguished as one of the largest and most evolved families among flowering plants (Hsiao, *et al.*, 2011). The orchid industry is economically important in Malaysia, Singapore, Thailand, China, Netherlands, Hawaii and continental USA (Hu, *et al.*, 1993; Eun and Wong, 1999; Chan, *et al.*, 2005; Khentry, *et al.*, 2006). This is due to orchid flower phenotype with the wide variety of colours, sizes and shapes, floriferous flower sprays, long flowering life of several weeks to months and its availability throughout the year. To meet consumer expectations, novel flower colours and shapes are very important to the horticultural industry. However development of new varieties of orchids with different colours and shapes is limited by the long breeding time required (usually three to four years for one generation). Based on these limitations, the development of time and cost effective approaches for manipulation of orchid flower colour and shape appears very attractive.

There are several factors to determine flower colour, which include secondary metabolites, pH value, metal ions, flavonoid co-pigments, environmental temperature and morphology of the epidermal cells (Baumann, *et al.*, 2007). Previous studies have shown that MYB transcription factors play a role in the regulation of plant development, hormone signalling and secondary metabolism (Espley, *et al.*, 2007). Some MYB families have been shown to regulate flower cell shape and pigments (Perez-Rodriguez, *et al.*, 2005; Baumann, *et al.*, 2007).

Several approaches have been carried out by the researchers in genetic of plants to improve its quality and quantity. Overexpression and RNA interference (RNAi) technology have been widely used in genetic engineering of secondary metabolites, biotic and abiotic stress tolerance and nutritional improvement of plants. In RNAi technology, it is the action of small interfering RNAs (siRNA) and microRNAs (miRNA) which result in gene silencing through cleavage of mRNAs and blockage of

protein synthesis. RNAi modification of a plant trait can be carried out by direct mechanical inoculation of dsRNA, which has been tested for plant virus resistance studies and also by transgenic approaches to introduce new DNA (encoding RNA silencing sequences) into plants either transiently or stably.

Among these, the direct mechanical inoculation of dsRNA is attractive as a rapid low cost method that has been effective to control the expression of viral genes via induced RNA silencing in plants. Therefore, this method was selected for application to silence an endogenous plant gene towards investigating the function of a MYB transcription factor in an orchid.

The aim of this thesis was to develop a simple and quick approach to modify orchid flower colour using *Dendrobium hybrida* as a model plant. The specific objectives were:

1. To isolate MYB gene sequences involved in pigment biosynthesis and cell shape development from *Dendrobium hybrida*.
2. To investigate the roles of the isolated *MYB* gene sequences by RNAi knock down of the expression of these genes using exogenously applied dsRNA.

Chapter 2.0 Literature review

2.1 Orchids

Orchidaceae, popularly known as orchids, are renowned for their diverse flower shapes and exotic colours (Hsiao, *et al.*, 2011). Orchid plants are found worldwide and show a wide diversity of epiphytic and terrestrial growth forms (Hsiao, *et al.*, 2011). The orchid industry is economically important in Malaysia, Singapore, Thailand, China, Netherlands, Hawaii and continental USA (Hu, *et al.*, 1993; Eun and Wong, 1999; Chan, *et al.*, 2005; Khentry, *et al.*, 2006). The *Orchidaceae* family comprises more than 25,000 species in approximately 900 different genera distributed worldwide and includes a economically important ornamental species from diverse genera such as *Cymbidium, Arachnis, Cattleya,, Ascocentrum, Dendrobium, Renanthera, Vanda, Oncidium, Laelia, Paphiopedilum, Phalaenopsis* and their intergeneric hybrids (Eun and Wong, 1999; Lee and Chang, 2008). Among them, *Dendrobium* (1,400 species) is the third largest genera (Pillon and Chase, 2007) and its popularity increasing in the international floricultural field (Kuehnle, 2007). This is due to their phenotype with a variety of colours, sizes and shapes, floriferous flower sprays, long flowering life of several weeks to months and its availability throughout the year (Kuehnle, 2007).

The cut flower industry in Malaysia is showing tremendous growth, especially in the last ten years, with flowers and ornamental plants being exported to other countries. Orchid growing is a multi-million ringgit industry with approximately 191.9 million stalks of orchid cut flowers and pot flowers produced in the year 2011. Furthermore, Malaysia exported about RM 109.1 million worth of orchids in 2011 as shown in table 1 (Ministry of Agriculture & Agro-based Industry, Malaysia, 2011).

Table 2.1. Quantity of orchid plants production and wholesale value of potted orchids in Malaysia, 2006-2011 (Ministry of Agriculture & Agro-based Industry, Malaysia, 2011).

Item	2006	2007	2008	2009	2010	2011
Number of Planted Flowers	12,800,670	13,312,697	27,536,379	35,722,042	40,128,642	42,033,745
Total Production (cuttings/pots/plants)	17,057,700	18,034,475	119,170,420	154,595,882	173,666,522	191,911,323
Total Production Value (RM)	10,462,520	10,881,022	83,419,295	108,217,119	104,199,913	109,146,794

2.2 Factors influencing flower colour

Flowers of orchid plants display diverse colour, pigmentation pattern, morphologies and pollination strategies (Mudalige-Jayawickrama, *et al.*, 2005; Albert, *et al.*, 2010). This diversity occurred due to evolutionary processes by successfully pollinators' reproduction or wind pollination (Schwinn, *et al.*, 2006). The colour signal of a flower is determined by the composition of different pigments produced in the petals, patterns of pigmentation, colour intensity, colour saturation and colour brightness (Lunau, 2000; Baumann, *et al.*, 2007). Colour changes of the flower can lead to the switch of the pollinator, hence, result in genetic isolation and ultimately speciation. For example, carotenoid change in *Mimulus* leads to the switch of pollinator from bumblebee to hummingbird (Bradshaw and Schemske, 2003; Koes, *et al.*, 2005).

2.2.1 Secondary metabolites and flower colour

Plant colours and pigmentation patterns are provided by secondary metabolites which are tetrapyrroles, flavonoids, carotenoids and betalains (Y. Tanaka, *et al.*, 2010; Miller, *et al.*, 2011; Kamiishi, *et al.*, 2012). Anthocyanins, which are derivative of flavonoids are accumulated at the adaxial epidermal layer, sub-epidermal layer or extended to the mesophyll layer if the colour intensity is high whilst carotenoids accumulate evenly in the adaxial epidermal layer, sub-epidermal layer and mesophyll layer (Mudalige, *et al.*, 2003). Therefore, modification of the metabolite contents in the cell causes variation in flower colour thus produces unique flower colour (Nishihara and Nakatsuka, 2011). Tetrapyrroles such as chlorophylls are all green and play a role in photosynthesis whereas flavonoids are water-soluble and phenolic molecules that occur in almost all vascular plants (Miller, *et al.*, 2011). Biosynthesis of flavonoid contributes pale yellow, orange, magenta, red, blue, pink and purple pigments in plants (Winkel-Shirley, 2001; Farzad, *et al.*, 2003; Wang, *et al.*, 2006; Ma, *et al.*, 2009; Y. Tanaka, *et al.*, 2010; Nishihara and Nakatsuka, 2011;). The derivatives of flavonoid are flavones,

flavonols, anthocyanins and isoflavonoids (Miller, *et al.*, 2011; Nishihara and Nakatsuka, 2011). A typical flavonoid biosynthesis pathway in higher plants is shown in figure 2.1 (Nishihara and Nakatsuka, 2011). Anthocyanins play the main role in colour development in plants (Albert, *et al.*, 2010). Winkel-Shirley, 2001 reported that anthocyanin biosynthetic genes that are involved in regulation of plant pigmentation are highly conserved in higher plants. The anthocyanin biosynthesis pathway has been well studied in Cymbidium (Albert, *et al.*, 2010), Phalaenopsis (Ma, *et al.*, 2009), Oncidium (Chiou and Yeh, 2008), Antirrhinum (Schwinn, *et al.*, 2006), Petunia (Quattrocchio, *et al.*, 1999; Quattrocchio, *et al.*, 2006), maize (Hernandez, *et al.*, 2004), Viola (Farzad, *et al.*, 2003) and Arabidopsis (Stracke, *et al.*, 2007).

Carotenoids which contribute yellow, red and orange colours are lipid soluble pigments that occur in non-photosynthetic chromoplasts in flowers and fruits (Miller, *et al.*, 2011). For instance, yellow coloration in *Dendrobium* and *Cattleya* alliance orchids has been attributed to carotenoids (Thammasiri, 1986). Lastly, the betalain biosynthetic pathway results in yellow and red colours. This pathway is less well studied due to it is only being present in few species of *Caryophyllales*. In addition, it is difficult to utilize for genetic engineering (Nishihara and Nakatsuka, 2011; Tanaka, *et al.*, 2010).

2.2.2 Cell shape and flower colour

Together with anthocyanins and carotenoids, flower colour is also influenced by factors such as shape of epidermal cells, temperature, co-pigments, vacuolar pH and metal ions (Kay, *et al.*, 1981; Winkel-Shirley, 2001; Hieber, *et al.*, 2006; Baumann, *et al.*, 2007; Y. Tanaka, *et al.*, 2010; Miller, *et al.*, 2011).

Different epidermal cell shapes, for instance, conical, flat or pointed can affect flower colour differently (Noda, *et al.*, 1994; Baumann, *et al.*, 2007; Miller, *et al.*, 2011). Much petal colour is localised in the vacuole of epidermal cells, hence, these cells play a crucial role in understanding the function of petals (Winkel-Shirley, 2001; Quintana,

et al., 2007; Miller, *et al.*, 2011; Tsuda, *et al.*, 2004). Epidermal cell shape is the first point of contact with both biotic and abiotic environments. This cell shape provides visual cues to pollinators and influences temperature and light capture of the plants (Miller, *et al.*, 2011; Whitney, *et al.*, 2011). Among different cell shapes, a conical cell shape could enhance light absorption by the pigments by increasing the amount of incident light that enters the epidermal cells (Yoshida, *et al.*, 2006; Baumann, *et al.*, 2007). Hence, it increases the chance of the flower being visited by a pollinator due to the petal presenting a larger surface area to approaching pollinators which enhanced the pollination success (Noda, *et al.*, 1994; Glover and Martin, 1998; Whitney, *et al.*, 2011). For instance, *Antirrhinum majus* flowers with conical epidermal cells are more attractive to pollinating bees when compared with flat petal epidermal cells (Comba, *et al.*, 2000; Baumann, *et al.*, 2007). Orchids, one of the deceptive plants, attract pollinators by mimicking food plants or female insects. Hence, flower colouration, morphology and odour play the key roles in deceiving pollinators (Schluter and Schiestl, 2008; Y. Tanaka, *et al.*, 2010).

2.2.3 Metal ions and flower colour

Furthermore, the iron ion content could influence flower colour. For instance, an iron transporter present in the vacuolar membrane in the epidermal cells of perianth bottom (*TgVit1*) and high concentrations of ferric ion were related to blue colouration in the lower perianth of *Tulipa gesneriana* flowers. The blue colouration in the petal of *Tulipa gesneriana* was developed by the ferric ion (Fe) complex of delphinidin 3-rutinoside (K. Momonoi, *et al.*, 2009; Shoji, *et al.*, 2010; Y. Tanaka, *et al.*, 2010; Kazumi Momonoi, *et al.*, 2012). Besides that, it was also supported by a study that a higher concentration of Al^{3+} was found in blue coloured sepals when compared with red coloured sepals in *Hydrangea macrophylla* (Ito, *et al.*, 2009). Thus, iron ions are essential components for blue colouration in flowers for the accumulation of

delphinidin and cyanidin chromophore pigments (Momonoi, *et al.*, 2009). For instance, blue colour in the Himalayan blue poppy *Meconopsis grandis* (Yoshida, *et al.*, 2006) and cornflower *Centaurea cyanus* (Kondo, *et al.*, 1998; Shiono, *et al.*, 2005).

2.2.4 Co-pigmentation and flower colour

Co-pigmentation is one of the factors that contribute to flower colour. Flavones and flavonols act as co-pigments and form complexes with anthocyanins, contributing to flower colour (Snieckus, 1983; Farzad, *et al.*, 2003; Nishihara and Nakatsuka, 2011). Flavones act as co-pigment and complexation with metal ions results in a bathochromic shift to a bluer or deeper flower colour (Tanaka, *et al.*, 2010). Moreover, coexisting metal ions and vacuolar pH are also play a key role in flower colouration. Lower pH (acidic) and higher pH or neutral of vacuolar pH give redder and bluer colours respectively (Fukada-Tanaka, *et al.*, 2000; M. Mori, *et al.*, 2009). For instance, intense red rose petals have a low pH (pH 4.0) (Tanaka, *et al.*, 2010). Studies of vacuolar pH of flower colour were done on *Ipomoea nil* and *Veronica persica* (Fukada-Tanaka, *et al.*, 2000; Yamaguchi, *et al.*, 2001). Mutation of a gene encoding a vacuolar Na^+/H^+-exchanger (*InNHX1*) interfered with the increase of vacuolar pH, thus, plants were unable to produce the normal bright blue petals of *Ipomoea nil* (Yamaguchi, *et al.*, 2001; Fukada, *et al.*, 2000).

2.2.5 Environmental temperature and flower colour

Environmental temperature can control the colour of flowers by altering the anthocyanin biosynthesis and thus content: dihydroflavonol 4-reductase (DFR), chalcone synthase (CHS), F3H (flavanone 3-hydroxylase) (Dela, *et al.*, 2003; K. Mori, *et al.*, 2007). Rises of temperature could cause chemical degradation, enzymatic degradation and polymerization with proanthocyanidin (Sipiora and Granda, 1998; K. Mori, *et al.*, 2007). A study on *Malus* x *domestica* Borkh fruit reported that anthocyanin concentrations were lower under hot climatic conditions due to high temperatures preventing the accumulation of cyanidin and UDP-sugars (Lin-Wang, *et al.*, 2011). Besides that, lower temperature caused higher or increased anthocyanin content whilst higher temperature resulted in loss or decrease of anthocyanin content in a wide range of species, including *Arabidopsis thaliana* (Rowan, *et al.*, 2009), *Citrus sinensis* (Lo, *et al.*, 2004), *Rosa hybrida* (Dela, *et al.*, 2003) and *Vitis vinifera* (Mori, *et al.*, 2007).

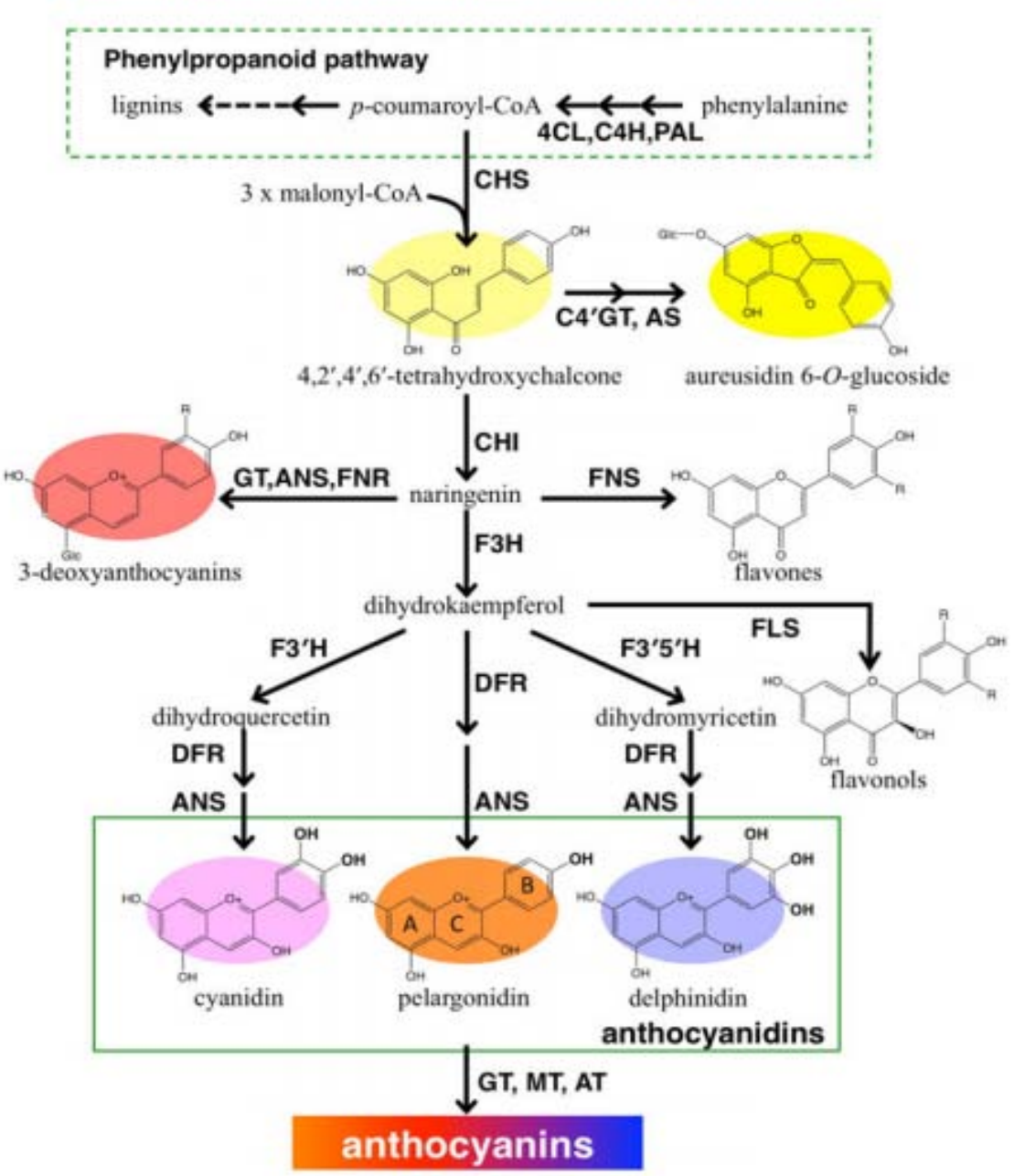


Figure 2.1: Flavonoid biosynthetic pathway and flavonoid compounds accumulated in flowers . A simplified pathway derived from several plant species is depicted for ease of explanation. The painted colours show image of each compound: light yellow: 4,2',4',6'-tetrahydroxychalcone; yellow: aureusidin 6 -O-glucoside; red: 3-deoxyanthocyanins; pink: cyaniding; orange: pelargonidin; purple: delphinidin. ANS: anthocyanidin synthase; AS: aureusidin synthase; AT: acyltransferase; C4'GT: chalcone 4'-O-glucosyltransferase; C4H: cinnamate-4-hydroxylase; CHI: chalcone isomerise; CHS: chalcone synthase; 4CL: 4-coumarate:CoA ligase; DFR: dihydroflavonol 4 -reductase; F3H:flavanone 3 -hydroxylase; F3'H:flavonoid 3' -hydroxylase; F3'5'H: flavonoid 3',5'-hydroxylase; FLS: flavonol synthase; FNR: flavanone 4-reductase; FNS: flavone synthase; GT: glycosyltransferase; MT: methyltransferase; PAL: phenylalanine ammonialyase (Nishihara and Nakatsuka, 2011, p. 435).

2.3 Strategies to modify flower colour

Flower colour is very important for plant breeders in the horticultural field. Traditional breeding was used for decades to breed different coloured cultivars by using genetically related species or natural mutants. However, there is some barrier to traditional techniques in producing some particular flower colours, for instance a blue rose and carnation, due to lack of delphinidin-based anthocyanins (Nishihara and Nakatsuka, 2011; Tanaka, *et al.*, 2010).

2.3.1 Genetic engineering of flavonoid pigments

Therefore, genetic engineering using transgenic approaches were studied to produce a novel flower colour (Tsuda, *et al*., 2004; S. Tanaka, *et al.*, 1997; Yoshikazu Tanaka, *et al.*, 2009; Boase, *et al.*, 2010; Y. Tanaka, *et al.*, 2010; Nishihara and Nakatsuka, 2011). Flavonoids are often the target to modify metabolic pathways as it's role is not critical in plant survival (Nishihara and Nakatsuka, 2011). In contrast, carotenoids are rather difficult to modify due to their role as the precursors for the synthesis of the plant hormones such as abscisic acid, strigolactone and gibberellins and it is also an essential component for photosynthesis (Grotewold, 2006; Y. Tanaka, *et al.*, 2010; Nishihara and Nakatsuka, 2011). Modification of the enzymes involved in the flavonoid pathway is an attractive metabolic engineering target that gives the chance of producing novel colour in ornamental plants (Nakatsuka, *et al.*, 2008; Boase, *et al.*, 2010). In previous studies on flower colour modification, particular attention has been focused on flavonoid 3' -hydroxylase; F3'5'H and flavonoid 3',5' -hydroxylase which are the enzymes that function for the hydroxylation of the B-ring of the flavonoid molecule (Figure. 2.1) due to their main role in colour formation of anthocyanin pigments (Katsumoto, *et al.*, 2007; Nakatsuka, *et al.*, 2008; Boase, *et al.*, 2010; Tsuda *et al*., 2004).

There are two main approaches to modify flower colour, which are endogenous silencing of flavonoid pigments and non-native accumulation of pigments in flower. These are aimed at producing paler flower colour and deeper flower colour, respectively (Nishihara and Nakatsuka, 2011). Meyer *et al*., 1987 were first reported the success of flower colour modification of mutant white flowered petunia to pale pink flower colour by over-expressing a heterologous maize *dihydroflavonol 4-reductase* (*DFR*, A1) gene. A more recent study showed that blue-hued *Rosa hybrida* flower was produced by heterologous expression of the *flavonoid 3',5'-hydoxylase* (*F3'5'H*) gene from *Viola* spp. (Katsumoto, *et al.*, 2007). Over-expression of *Lotus japonicus polyketide reductase* (*PKR*) and *Zea mays dihydroflavonol 4-reductase (DFR)* gene in *Petunia hybrida* caused the flower colour to change from red to variegated red and pale pink to brick-red, respectively (Shimada et al., 2006; Meyer *et al*. 1987).

2.3.2 Antisense mediated suppression technology

In the endogenous silencing approach, or antisense suppression was preliminary used in flower colour modification by van der Krol *et al*., 1988 to produce white flowers in Petunia and tobacco by constitutive expression of an antisense *chalcone synthase* (*CHS*) gene. White flowers were similarly produced in other plants such as *Gentiana sp, , Gerbera hybrida*, *Chrysanthemum cv. Moneymaker* and *Eustoma grandiflorum* via antisense mediated suppression of endogenous *chalcone synthase* (*CHS*) (Nishihara, *et al*., 2006; Elomaa, *et al.*, 1993; Deroles, *et al.*, 1998; Katsumoto, *et al.*, 2007; Tsuda, *et al*., 2004). Other than flower colour modification, antisense mediated suppression also caused fruit colour modification in *Fragaria* × *ananassa*, from bright red to pink by suppression of *chalcone synthase* (*CHS*) (Lunkenbein, *et al.*, 2006). Antisense suppression of *DFR* gene in *Petunia hybrida* also produced a paler flower colour (Tsuda, *et al*., 2004).

2.3.3 Post-transcriptional gene silencing and RNA interference technology

The next technology to be developed for flower colour modification was post-transcriptional gene silencing (PTGS) or cosuppresion. For instance, cosuppression of exogenous *CHS* caused white colour flower in *Nicotiana tabacum* cv. and Petunia plants (Wang, *et al.*, 2006). Subsequently, RNA interference technique (RNAi) was developed to modify flower colour. Studies showed that interference (RNAi)-mediated *Gentiana sp* and *Petunia hybrida* produced white colour flower due to RNAi mediated suppression of *chalcone synthase* (*CHS*) (Nakatsuka, *et al.*, 2008; Tanaka, *et al.*, 1998). However, RNA interference of endogenous *anthocyanidin synthase* (*ANS*) and *flavonoid 3',5'-hydroxylase* (*F3'5'H*) caused the modification of purple *Gentian sp.* to pale blue and magenta, respectively (Nakatsuka, *et al*., 2010; Nakatsuka, *et al*., 2008). Orange flowers were obtained by RNAi suppression of *flavonoid 3'-hydroxylase* (*F3'H*) gene in purple flower coloured *Petunia hybrida* (Tsuda, *et al*., 2004). Examples of successful modified flower colour in different plants by each strategy are summarized in table 2.2.

It has been suggested that regulation of regulatory genes could co-ordinate control the metabolic flux when compared with the modification of single enzymatic step (Broun, 2004). For example, *GhMYB10* was identified to induce cyanidin biosynthesis in undifferentiated callus and vegetative and enhanced pelargonidin production in floral tissues (Laitinen, *et al.*, 2008). This suggests that it is a transcription factor that activates a subset of structural anthocyanin genes (Quattrocchio, *et al.*, 1999).

Table 2.2. Examples of successful modified flower colour in different plants by regulating flavonoid biosynthesis pathway

Plant species	Original colours	Gene sources	Methods	Produced flower colours	References
Petunia hybrida	Blue	*Mazus pumilum CHS*	Dominant negative	Pale blue	Hanumappa, *et al.*, 2007
	Red	*Lotus japonicus PKR*	Over expression	Variegated red	Shimada, *et al*.,2006
	Dark purple	Endogenous *F3H*	Sense	Star shape pale purple	Tsuda, *et al*.,2004
	Dark purple	Endogenous *DFR*	Antisense	Pale purple	
	Dark purple	Endogenous *F3'5'H*	Sense	Pink (resembled flowers of Surfinia Hot Pink)	
	Dark purple	Endogenous *FLS, AR-AT* and *F3'5'H*	Sense	Red	
	Pale pink	*Zea mays DFR*	Over expression	Brick-red flowers	Meyer, *et al*.,1987
Tricyrtis sp.	Reddish-purple spots	Endogenous *CHS*	RNAi	White	Kamiishi, *et al.*, 2012
Rosa hybrida	Magenta	*Viola* spp *F3'5'H*	Over expression	Blue	Katsumoto, *et al.*, 2007
Gentiana sp.	Blue	Endogenous *CHS*	RNAi	Pale blue to white	Nakatsuka, *et al*., 2008
	Blue	Endogenous *ANS*	RNAi	Pale blue	Nakatsuka, *et al*., 2008
	Blue	Endogenous *F3'5'H*	RNAi	Magenta	Nakatsuka, *et al*., 2008
	Blue	Endogenous *CHS*	Antisense	White	Nishihara, *et al.*, 2005
Torenia hybrida	Blue	*Antirrhinum majus AS*	Over expression	Yellow	Ono, *et al*., 2006
Cyclamen persicum	Purple	Endogenous *F3'5'H*	Antisense	Red to pink	Boase, *et al*., 2010
Ostespermum hybrida	Magenta	Endogenous *F3'5'H*	RNAi	Reddish	Seitz, *et al.*, 2007
		Gerbera hybrida DFR	Over expression		
Fragaria × *ananassa*	Bright red	Endogenous *CHS*	Antisense	Pink	Lunkenbein, *et al.*, 2006

2.4 MYB Transcription Factors

Transcription factors (sometimes called sequence-specific DNA-binding factors) are proteins that bind to DNA *cis*-acting elements in the promoters that they target. By binding to specific DNA sequences, they activate or repress the production of mRNA transcripts from genes (Stracke, *et al.*, 2001; Y. H. Chen, *et al.*, 2006). Transcription factors from within and between species that share some conserved region in their DNA binding domains can be grouped into families (Liu, *et al.*, 1999). Plant MYB genes can be divided into subgroups based on conserved regions of the sequence. Most of the genes from the same subgroup share similar biochemical functions (Baumann, *et al.*, 2007; Jaffe, *et al.*, 2007). For instance, plant MYB transcription factors in subgroup 9 play a key role in conical-papillate cell formation in the petal epidermis (Jaffe, *et al.*, 2007). Structural similarity between *MIXTA*, *PhMYB1* and *AtMYB16* has led to the claims of orthology and functional equivalence of these genes (van Houwelingen, *et al.*, 1998; Baumann, *et al.*, 2007; Jaffe, *et al.*, 2007). However, there can be exceptions where the biological function of proteins in same subgroup vary (Liu, *et al.*, 1999).

The discovery of the v-myb oncogene of the avian myeloblastosis virus led to the discovery of a large family of MYB-related genes in the eukaryotes. The MYB domain of the MYB proteins is conserved amongst animals, plants and yeast (Lipsick, 1996). The MYB superfamily consists of six subfamilies which are R1R2R3, R2R3, CAPRICE-like, GARP, SHAQKY and telomere binding protein-like. All of the subfamilies posses a particular number of MYB motifs (Bailey, *et al.*, 2008). For instance, R2R3 MYB subfamily members have a MYB domain which is constituted by two imperfect repeats of approximately 50-53 amino acids. Each repeat posseses three regularly spaced tryptophan residues which encode three α-helices with the second and third helix forming a helix–turn–helix conformation to bind to the target DNA (Jaffe, *et al.*, 2007; Ogata, *et al.*, 1994; Stracke, *et al.*, 2001; Lipsick, 1996). However, there are

some exceptions where a phenylalanine residue replaces the first tryptophan in the R3 repeat (Stracke, *et al.*, 2001; Konig, *et al.*, 1996). Most of the plant MYB genes belong to the R2R3 subfamily, which are most similar to R2 and R3 repeats of the animal c-MYB proteins (Jaffe, *et al.*, 2007; Stracke, *et al.*, 2001; Kranz, *et al.*, 1998). MYB proteins are identified by an N-terminal DNA binding domain due to high conservation among plants whereas the C-terminal of the R2R3-MYB, which is the activation domain of the MYB varies (Matsuda, *et al.*, 2011; Kranz, *et al.*, 1998; Lipsick, 1996). The first plant MYB gene discovered was *C1* from *Zea mays* which plays a role in synthesis of anthocyanin in the aleurone of the *Zea mays* kernels (Gonzalez, *et al.*, 2008; Cone, *et al.*, 1986). After this discovery, the known function of plant MYB genes increased widely. MYB genes are specifically expressed in different tissues and physiological conditions, this raised the idea that they are involved in various regulatory processes (Kranz, *et al.*, 1998).

As plants live a sessile life style they have evolved large families of transcription factors to accommodate the demand for developmental processes and defense responses.. For instance, approximately 6% of the *Arabidopsis thaliana* genome is encoded by transcription factor genes and 9% from the total transcription factor genes are members of the MYB family (Riechmann, *et al.*, 2000). In plants, MYB genes are well documented, 126 members of *MYB* genes in *Arabidopsis* (Stracke, *et al.*, 2001; Zimmermann, *et al.*, 2004), 183 members in *Oryza sativa* (Y. H. Chen, *et al.*, 2006) and 192 members in *Populus trichocarpa* (Wilkins, *et al.*, 2009). There are 24 subgroups in the *Arabidopsis thaliana R2R3-MYB* gene family, with each subgroup possessing specific motifs in the C-terminal region (Kranz, *et al.*, 1998; Stracke, *et al.*, 2001; Matsuda, *et al.*, 2011).

Plant *MYB* genes have been discovered to play a role in the regulation of plant development, hormone signalling and secondary metabolism (Espley, *et al.*, 2007).

Plant development roles include the development of root hairs, trichomes and seed coat mucilage (Laitinen, *et al.*, 2008; Di Stilio, *et al.*, 2009; Baumann, *et al.*, 2007; Jaffe, *et al.*, 2007). Some of *MYB* genes are involved in the pathways of phenylpropanoid biosynthesis (Matsuda, *et al.*, 2011; Schwinn, *et al.*, 2006; Zimmermann, *et al.*, 2004; deVetten, *et al.*, 1997) and signaling pathways that are activated to respond to environmental stresses such as drought, cold weather, ultraviolet (UV)-B irradiation, and rhizobacteria-mediated induced systemic resistance (Matsuda, *et al.*, 2011; Zhu, *et al.*, 2005; Van der Ent, *et al.*, 2008). A complex constituted by three transcription factors, which are *R2R3-MYB*, basic helix loop helix (*bHLH*) and WD4-repeat protein (MBW complex) activates the transcription of flavonoid biosynthesis gene (Stracke, *et al.*, 2007; Gonzalez, *et al.*, 2008; Jaffe, *et al.*, 2007; Schwinn, *et al.*, 2006; Koes, *et al.*, 2005; Ramsay and Glover, 2005).

Previous studies showed the process of regulation of *R2R3-MYB* in model species such as *Petunia hybrida* (Avila, *et al.*, 1993; Quattrocchio, *et al.*, 2006), *Arabidopsis thaliana* (Gonzalez, *et al.*, 2008; Jakoby, *et al.*, 2008; Stracke, *et al.*, 2001; Kranz, *et al.*, 1998), *Antirrhinum majus* (Baumann, *et al.*, 2007; Schwinn, *et al.*, 2006; Perez-Rodriguez, *et al.*, 2005), *Malus domestica* (Takos, *et al.*, 2006; Ban, *et al.*, 2007; Espley, *et al.*, 2007), *Gossypium hirsutum* (Machado, *et al.*, 2009), *Fagus crenata* (Matsuda, *et al.*, 2011), *Gerbera hybrida* (Laitinen, *et al.*, 2008), *Vitis vinifera* (Azuma, *et al.*, 2009), *Lotus japonicas* (Yoshida, *et al.*, 2006), *Thalictrum thalictroides* (Di Stilio, *et al.*, 2009), *Pyrus pyrifolia* (Feng, *et al.*, 2010), *Cymbidium hybrid*a (Albert, *et al.*, 2010), *Phalaenopsis amabilis* (Ma, *et al.*, 2009) and *Oncidium Gower Ramsey* (Chiou and Yeh, 2008). *AmMIXTA* and *PhMYB1* genes control cell differentiation in *Antirrhinum majus* and could activate anthocyanin biosynthetic gene expression (Avila, *et al.*, 1993; Noda, *et al.*, 1994) whereas *AmMYBML1* induced the production of both trichomes and conical cells on floral tissues (Perez-Rodriguez, *et al.*, 2005). The

monocot *Dendrobium DwMYB* proteins were discovered to be more similar to dicotyledous *Arabidopsis* MYB proteins than those from monocots, such as maize and rice (Wu, *et al.*, 2003). The function of some plant *R2R3-MYB* genes are summarized in table 2.3.

Table 2.3. Examples of *R2R3-MYB* genes and its function in agricultural plants

R2R3			
Plant species	Plant *MYB* gene	Biological functions	Reference
Arabidopsis	*AtMYB75/production of anthocyanin pigment1 (PAP1)*	Anthocyanin regulator in seedlings Anthocyanin regulator in vegetative organ	Teng, *et al.*, 2005 Borevitz, *et al.*, 2000
	AtMYB90/PAP2	Control anthocyanin production	Borevitz, *et al.*, 2000
	AtMYB4	Represses the synthesis of sinapoyl malate	Jin, *et al.*, 2000
	AtMYB34/ATR1 (ALTERED TRYPTOPHAN REGULATION1)	Regulator of tryptophan biosynthesis	Bender and Fink, 1998
	AtMYB12	Flavonol-specific activator of flavonoid biosynthesis primarily in root	Mehrtens, *et al.*, 2005
	AtMYB111	Controls flavonol biosynthesis primarily in cotyledons	Stracke, *et al.*, 2007
	AtMYB23	Trichome initiation	Kirik, *et al.*, 2001
	AtMYBGL1	Trichome initiation	Kirik, *et al.*, 2001
	AtMYB16	Extending the growth of conical-papillate petal epidermal cells	Baumann, *et al.*, 2007
	AtMYB106	Trichome formation	Jakoby, *et al.*, 2008
Trifolium arvense	*TaMYB14*	Proanthocyanidins biosynthesis	Hancock, *et al.*, 2012
Zea mays	*C1*	Anthocyanin biosynthesis in the aleurone layer of the endosperm	Cone, *et al.*, 1986
Zea mays	*p1*	Anthocyanin regulator in vegetative and floral tissues	Cone, *et al.*, 1993
Antirrhinum	*Rosea1*	Activation of expression of F3H, F3'H, FLS, DFR, and UFGT in flower and stem tissues	Schwinn, *et al.*, 2006
	Rosea2	Activation of expression of F3'H	Schwinn, *et al.*, 2006
	Venosa	Activation of expression CHI, F3H, F3'H, FLS, ANS, UFGT, and AT	Schwinn, *et al.*, 2006

Table 2.3 continued. Examples of *R2R3-MYB* genes and its function in agricultural plants

R2R3			
Plant species	Plant MYB	Biological functions	Reference
Antirrhinum	*AmMYBMX*	Regulating the differentiation of conical-papillate petal epidermal cells and initiate trichome development	Noda, *et al.*, 1994
	AmMYBML1	Differentiation of several petal epidermal cell types	Perez-Rodriguez, *et al.*, 2005
	AmMYBML2	Extending the growth of conical-papillate petal epidermal cells	Baumann, *et al.*, 2007
	AmMYBML3	Enhance cellular out-growth from the epidermis of all aerial organs	Jaffe, *et al.*, 2007
Petunia	*PhMYB1*	Extending the growth of conical-papillate petal epidermal cells	Avila, *et al.*, 1993; Baumann, *et al.*, 2007
	an2	Control anthocyanin production in flower	deVetten, *et al.*, 1997
Malus domestica	*MdMYBA*	Induced anthocyanin accumulation	Ban, *et al.*, 2007
	MdMYB1	Regulates structural genes across the flavonoid pathway in apple fruit, determining skin colour and providing the potential to modulate fruit colour	Takos, *et al.*, 2006
	MdMYB10	Induced anthocyanin accumulation	Espley, *et al.*, 2007
Gossypium hirsutum L	*GhMYB25*	Regulating specialized outgrowths of epidermal cells and cotton fibres	Machado, *et al.*, 2009
Gerbera hybrida	*GMYB10*	Regulate the accumulation of cyanidin in vegetative organs	Laitinen, *et al.*, 2008
Vitis vinifera	*VvmybA1*	Differential regulation of the anthocyanin biosynthesis pathway genes	Azuma, *et al.*, 2009
Thalictrum thalictroides	*TtMYBML2*	Promote conical cell growth in the petal and carpel epidermal cells	Di Stilio, *et al.*, 2009
Pyrus pyrifolia	*PyMYB10*	Regulate anthocyanin biosynthesis	Feng, *et al.*, 2010
Oncidium Gower Ramsey	*OgMYB1*	Regulate formation of red pigments through the activation of *OgCHI* and *OgDFR* transcription	Chiou and Yeh, 2008

Table 2.3 continued. Examples of *R2R3-MYB* genes and its function in agricultural plants

bHLH			
Zea mays	*R*	Regulate flavonoid pigments in parallel with the MYB proteins	Ludwig, *et al.*, 1989
	B	Activate transcription of maize flavonoid biosynthetic genes	Mol, *et al.*, 1996
Arabidopsis	*R/B like*	Control phenylpropanoid biosynthetic pathway and epidermal patterning in trichome and root hair development	Zimmermann, *et al.*, 2004
Petunia	*an1*	Control anthocyanin production in flower	deVetten, *et al.*, 1997
Antirrhinum	*Delila*	Activation of expression of the late biosynthetic genes (including F3H, DFR, AS, and UDP-glucose 3-O-flavonoid transferase (UFGT)	Martin, *et al.*, 1991
WD40			
Arabidopsis	*TTG1*	Control anthocyanin production, formation of hairs on leaves, stems, roots and the production of seed mucilage	Walker, *et al.*, 1999
Petunia	*AN 11*	Control anthocyanin production	deVetten, *et al.*, 1997
Antirrhinum	*pac1*	Control the levels of anthocyanin production in kernels	Carey, *et al.*, 2004

2.5 RNA silencing in plants

2.5.1 RNA silencing pathways in plants

RNA silencing was first discovered in *Petunia hybrida* plants (Jagtap, *et al.*, 2011 Herr and Baulcombe, 2004;). The researchers introduced transgene copies of flower pigmentation gene, *chalcone synthase* (*CHS*) into *Petunia hybrida* flowers with the idea to produce a more intense purple colouration in this flower. However, this introduction produced intense purple, patterns of purple and white, and flowers that were completely white (Napoli, *et al.*, 1990). Later, Lindbo and colleagues transformed tobacco plants with the gene sequence of the tobacco etch virus (TEV) coat protein (CP) which was able to provide TEV resistance to the plants (Lindbo, *et al.*, 1993). These studies revealed that in plants, introduced and endogenous gene were targeted specifically and were silenced to differing degrees (Lindbo, *et al.*, 1993; Napoli, *et al.*, 1990). Subsequently, endogenous small RNAs (siRNAs and miRNAs) were discovered in plants in 2002 (Llave, *et al.*, 2002; Reinhart, *et al.*, 2002). The major frameworks related to the biogenesis of miRNAs and siRNAs have been well established in the past ten years (X. M. Chen, 2012).

There are at least three RNA silencing pathways to silence target genes in plants. These are cytoplasmic siRNA silencing, endogenous mRNA cleavage by miRNAs and DNA methylation (Baulcombe, 2004; Ji and Chen, 2012). These silencing pathways play important biological roles in plant viral resistance, regulation of gene expression, condensation of chromatin into heterochromatin and protection of the genome from transposons (Ji and Chen, 2012; Jagtap, *et al.*, 2011; Baulcombe, 2004; Herr and Baulcombe, 2004).

2.5.1.1 Cytoplasmic siRNA silencing

Cytoplasmic siRNA silencing plays a main role in virus resistance and homology-dependent gene silencing in plants (Ji and Chen, 2012; Jagtap, *et al.*, 2011). The steps in this silencing pathway involve a few components which are; double stranded RNA (dsRNA), an enzyme called Dicer that has RNase III domains (DCL1) and AGRONAUTE1 protein (AGO1) (Dunoyer, *et al.*, 2010). dsRNA was discovered to act as an initiator for gene silencing in nematodes (Fire, *et al.*, 1998), plants (Waterhouse, *et al.*, 1998) and insects (Kennerdell and Carthew, 1998). The introduction of exogenous nucleic acid into the plant cell can cause the production of a dsRNA molecule. Subsequently, this dsRNA molecule is processed by DCL1 into typically 21 to 24 nucleotide length small RNA (sRNA) called short interfering RNA (siRNA) (X. M. Chen, 2009; Xie, *et al.*, 2010; Bernstein, *et al.*, 2001). AGO1 was discovered to use the guide strand of siRNA to target complementary mRNAs and to cleavage the target gene resulting in repression of gene expression (Baumberger and Baulcombe, 2005; Rand, *et al*., 2004). Moreover, Hammond and colleagues showed that the RNase III-like enzyme Dicer in *Drosophila melanogaster* cleaves the dsRNA into siRNA of approximately 21 to 25 nucleotides. This suggests that the mechanism is conserved across kingdom. A model of the cytoplasmic siRNA silencing pathway is shown in figure 2.

2.5.1.2 Endogenous mRNA cleavage by miRNAs

In nature, plant miRNAs target endogenous mRNA in a sequence specific manner to affect development and responses to environmental stimuli (Ji and Chen, 2012; Bartel, 2007). miRNAs which are short 21-24 nucleotide RNAs, are processed from single-stranded RNA transcripts that have the ability to produce imperfect double stranded stem loop precursor structure (Xie, *et al*., 2010; Ambros and Chen, 2007). The first miRNA (lin-4) was discovered in *Caenorhabditis elegans* (R. C. Lee, *et al.*, 1993).

Following this discovery, several hundred miRNAs were discovered in the model plant, *Arabidopsis thaliana* (Millar and Waterhouse, 2005). In the nucleus of *Arabidopsis*, an miRNA duplex is formed by the cleavage of the miRNA precursor with DCL1 and double-stranded RNA-binding domain (dsRBP) (Jover-Gil, *et al.*, 2012; Xie, *et al*., 2010). However, sRNA-specific methyltransferase HUA ENHANCER1 (HEN1) is essential to methylate 2' OH of the 3' terminal ribose of miRNA duplexes and to then protect these from polyuridylation and 3'-5' degradation (Ji and Chen, 2012; Yu, *et al.*, 2005; Chen, *et al*., 2002). Then, the miRNA duplex is transported to the cytoplasm via Exportin-5 ortholog HASTY (HST) (Park, et al., 2002). In the cytoplasm, a plant RNA-induced silencing complexes (RISC), which includes the RNase AGO1guides the cleavage activity of the target mRNA to negatively regulate gene expression or binds to the mRNA to halt protein translation (Huntzinger and Izaurralde, 2011; Baulcombe, 2004).

2.5.1.3 DNA methylation and transcriptional gene silencing (TGS)

The third pathway of RNA silencing in plants is related to epigenetic mechanisms which include DNA methylation and transcriptional gene silencing (TGS) (reviewed by He, *et al*., 2011; Mansoor, *et al*., 2006). DNA methylation is conserved in most major eukaryotic groups, including plants, and most of the fungi and animals (reviewed by He, *et al*., 2011). Approximately one-third of methylated DNA loci are rich in siRNAs. This supported that siRNAs play an important role in DNA methylation (Lister, *et al*., 2008). In plants, RNA-directed DNA methylation (RdDM) is a conserved de novo DNA methylation mechanism. This was first found in Wassenegger and colleagues' study on the transgenic potato containing viroid genes in 1994. They showed that replication of RNA viroid caused methylation of recombinant viroid sequence in the plant genome. Next, RdDM was recognized as TGS mechanism in plants. This mechanism involved diverse epigenetic phenomena, which include

transposon suppression, transgene silencing, paramutation and gene imprinting (Law and Jacobsen, 2010; Zaratiegui, *et al*., 2007).

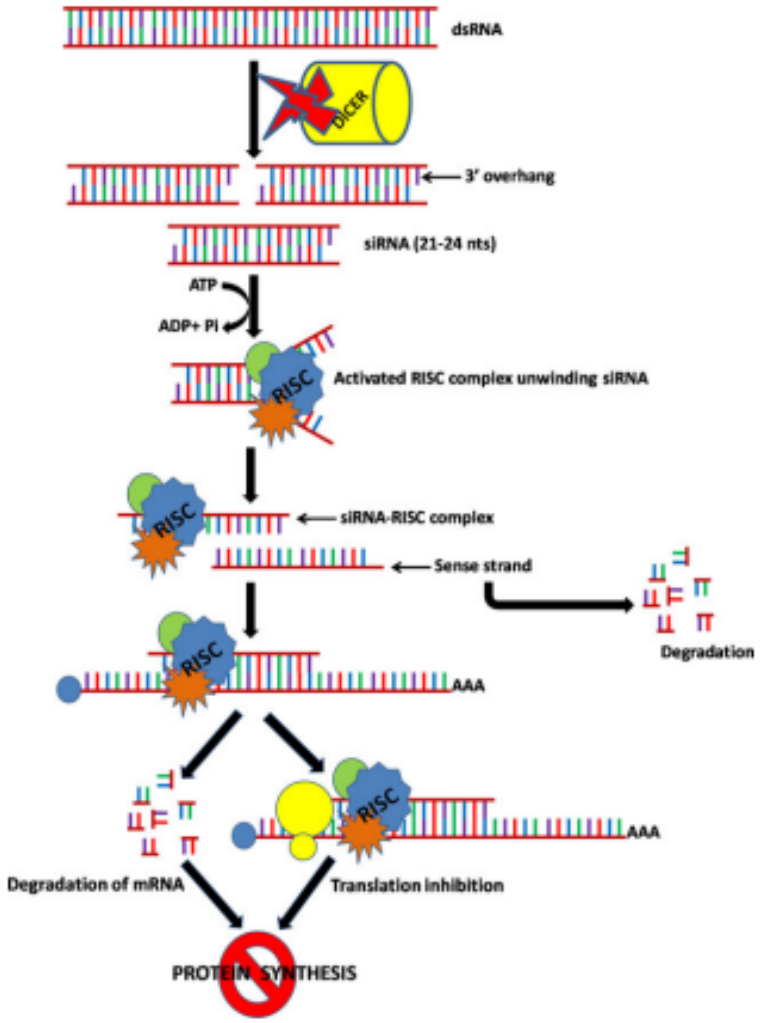


Figure 2.2: The RNAi mechanism-dsRNA is processed by DICER RNase III into 21–24 nt siRNA duplexes. The siRNAs are then incorporated into RISC. The siRNA–RISC complex then targets a sequence, complementary to the siRNA, in a piece of mRNA. The protein synthesis is blocked either by degradation of mRNA or inhibition of translation (Jagtap, *et al.*, 2011, p. 476).

2.5.2 Initiation and amplification of silencing

RNA-dependent RNA polymerases (RdRp) are required for RNA silencing in diverse organisms such as plants (Jeremy, *et al*., 2012; Li, *et al*., 2012; Dalmay, *et al*., 2000), *Caenorhabditis elegans* (Thivierge, *et al*., 2012; Sijen, *et al*. 2001) and fungi (Calo, *et al*., 2012; Volpe, *et al*. 2002). First plant RdRp (leRDR1) was purified from tomato (*Lycopersicon esculentum*) by Schiebel and colleagues in 1993. RdRp was studied for their diverse roles included antiviral defense and endogenous functions in plants. Endogenous functions including regulation of cellular gene expression and the control of chromatin structure (Li, *et al*., 2012; reviewed by Voinnet, 2008; Wang and Metzlaff, 2005). Their activities revealed the importance of small amounts of 'trigger' RNA, including transposon-derived and viral-derived sRNAs in RNA silencing (reviewed by Voinnet, 2008). These enzymes can mediate primer-dependent and primer-independent mechanisms of RNA silencing. Primer-dependent process uses the primary siRNA as a primer to produce dsRNA thus accelerate the production of dsRNA as well as siRNA (Birchler, 2009: Moissiard, et al., 2007; Herr and Baulcombe, 2004). On the other hand, the primer-independent process is important for the production of dsRNA from a single stranded RNA template and primary siRNA produced by DCL dicer (reviewed by Molnar, *et al*., 2011; Baulcombe , 2007; Baulcombe, 2004).

2.5.3 Mobile silencing signals

Gene silencing is triggered locally but can be spread throughout the plant by movement of mobile silencing signals between plant cells and through the vascular system of the plant (reviewed by Molnar, *et al*., 2011; Herr and Baulcombe, 2004; Fagard and Vaucheret, 2000; Palauqui, *et al*., 1997). This was supported by the experiment done by Herr and Baulcombe in 2004 in which the upper leaf of *Nicotiana benthamiana* showed loss of GFP after transient expression of a GFP transgene was

introduced into a lower leaf. Moreover, these mobile silencing signals also explained the observation of purple and white petunia flower colour pattern in *Chalcone synthase* cosuppression transgenic petunia (Herr and Baulcombe, 2004; Jorgensen, *et al*., 1995). Other than plants, mobile silencing signals also exist in animal such as *Caenorhabditis elegans*. However the mechanism is not the same due to animal cells are not interconnected and requires a putative RNA transporter protein (Feinberg and Hunter, 2003; Haywood, *et al*., 2002).

2.6 double-stranded RNA (dsRNA) as an analysis tool

Experimental induction of RNA silencing was successfully reported for diverse organisms such as *Caenorhabditis elegans* (Fire, *et al*., 1998; Timmons, *et al*., 2001), *Drosophila melanogaster* (Bernstein, *et al*., 2001; Hammond, *et al*., 2000), *Nicotiana benthamiana* (Tenllado, *et al*., 2003) and *Nicotiana tabacum* (Sun, *et al*., 2010) by introduction of dsRNA exogenously. RNA interference (RNAi) was first reported in *Caenorhabditis elegans*, where injection of dsRNA caused post-transcriptional gene silencing (PTGS) of a homologous sequence (Fire, *et al*., 1998). Subsequently, RNAi mediated by exogenous dsRNA has become an effective and time saving genetic tool for gene functional study and virus resistance in plants (Tenllado, *et al*., 2003; Sun, *et al*., 2010) when compared with regenerating transgenic plants.

For instance, dsRNA triggered from Pepper mild mottle virus (PMMoV) sequences can protect *Nicotiana benthamiana* from this virus infection by delivering in-vitro transcribed PMMoV dsRNA into plant leaf cells by mechanical inoculation (Tenllado, *et al*., 2003). Previous studies reported that the input dsRNA was relatively stable in the host up to several days post treatment (Tenllado and Diaz, 2001; Tenllado, *et al*., 2003; Sun, *et al*., 2010). Moreover, dsRNA that triggered genetic interference processes has

the ability to interfere in cells that are far distant from the initial site of dsRNA delivery (Fire, *et al*., 1998; Tenllado, *et al*., 2003).

Chapter 3.0 Materials and Methods

3.1 Research materials

3.1.1 Orchid plants

Dendrobium hybrida from a cross of *Dendrobium bobby messina* and *Dendrobium chao phraya* were used in this experiment. These plants were bought from Cheah Wah Sang Orchid Farm in Shah Alam, Selangor, Malaysia and grown in pots with charcoal as medium for root system.

3.2 Research methodology

3.2.1 Plant growth

Orchid plants were grown in the green house and watered twice a day and temperature was maintained at 25 ºC with 16 hours of light and 8 hours of dark photoperiod cycle.

3.2.2 Primer design

3.2.2.1 Degenerate primer design

To amplify the genes that may play a role in flower pigmentation and cell shape of *Dendrobium hybrida*, two pairs of degenerate primers were designed. These primer pairs were designed from two subgroups of *MYB* genes. One pair of primers named Dh1-MYB was designed from multiple alignments of *AtMYB75/PAP1* (accession number: NM_104541.3), *AtMYB90/PAP2* (accession number: NM_105310.3) and *PhMYBAN2* (accession number: AF146704.1) by using Bioedit software. A second pair of primers named Dh2-MYB was designed from multiple alignments of *AmMIXTA* (accession number: X79108.1), *AmMYBML1* (accession number: AJ006292.1) and *PhMYB1* (accession number: Z13996.1) using Bioedit software

(www.mbio.ncsu.edu/bioedit/bioedit.html). The sequences of primers were shown as in table below.

Table 3.1: Degenerate primer sequences

Dh1-MYB F	5’ AAA GGT GCW TGG ACY RMK GAA GAA GA 3’
Dh1-MYB R	5’ AGR TGR GTG TTC CAR TAR TTY TTG ACR TC 3’
Dh2-MYB F	5’ AGG GCC ATG GAC WSY AGA MGA AGA YC 3’
Dh2-MYB R	5’ GAA CTA YTG GAA CAC WCA TYT AAA GAA RCG 3’

3.2.2.2 Specific primer design

Specific primers were designed using Primer3 Input (version 0.4.0) (http://frodo.wi.mit.edu/primer3/), FastPCR software (http://en.bio-soft.net/pcr/FastPCR.html) and Primer Express® Software v2.0. Designed primers were analyzed by Gene Runner (www.generunner.net/). The list of primer sequences is shown in table 3.2.

Table 3.2: Specific primer sequences

Actin F	5’ TGG GCA CCT AAA TCT CTC AGC 3’
Actin R	5’ GTC AGG GAC ATC AAG GAG AAG 3’
Mixta 3’ RACE F	5’ GCT TGC ATA CAT TGA GAA GCA TGG GC 3’
Mixta 5’ RACE R	5’ AGA CCA CCT GTT GCC AAG GA 3’
NotI Mixta F	5’ AAA GCG GCC GCC CTA GGA AGA GGA TAC ACC ATT GAC 3’
NcoI Mixta R	5’ GGA CCC ATG GGG TGG CGG TGA ATT TGG A 3’
Mixta qPCR F	5’ TGC TGT CGG ATA AAG CCA ATG 3’
Mixta qPCR R	5’ GGT GGC GGT GAA TTT GGA 3’

3.2.3 Nucleic acid extraction

3.2.3.1 Small scale genomic DNA isolation using CTAB method

Two hundred and fifty mg of ground tissue was added to 1.5 ml microcentrifuge tubes containing 1 ml preheated extraction buffer (2 % CTAB, 100mM Tris-HCI pH 8, 20 mM EDTA, 2 % PVP 40, 1.4 M NaCl and 2 % β-mercaptoethanol). Extraction buffer was preheated for 10 minutes at 65 ˚C. After that, 500 µL of cold CI (24:1 chloroform: isoamylalcohol) was added then mixed gently for 10 minutes. The mixture was inverted for 5 minutes. It was centrifuged 5,031 x *g* for 5 minutes at room temperature and then the upper layer was transferred to new tube. An equal volume of cold isopropanol was added and incubated at -20 ˚C for 2 hours. After incubation, the tube was centrifuged 3,220 x *g* for 20 minutes at room temperature, the supernatant was discarded. The pellet was washed by using 1 ml 70 % ethanol and proceeded by centrifuging 5,031 x *g* for 5 minutes at room temperature. The supernatant was discarded and the pellet was dried using vacuum centrifugation PLUS HETO (Laboratory Equipment Solutions, Canada) for 2 minutes. Subsequently, the pellet was dissolved in 35 µl distilled water. Next, 100 µg/ml of RNase A was added to the dissolved DNA and incubated at 37 ˚C for 30 minutes. DNA solutions were stored at -20 ˚C (method modified from Doyle & Doyle, 1990). Finally, extracted DNA was visualized by staining with 0.5 µg/ml of Ethidium bromide on a 1 % agarose gel (The method was as shown in 3.2.18).

3.2.3.2 Total RNA extraction (CTAB method)

One g of sample was weighed and placed immediately in liquid nitrogen and ground thoroughly with a mortar and pestle. The powdered tissue was then placed in a 2 ml microcentrifuge tube and 1.5 ml of pre-warmed (65 °C) extraction buffer (100 mM Tris-HCI pH 8, 25 mM EDTA, 2 M NaCI, 2 % CTAB w/v, 2 % PVP 10 w/v and 2 % ß-

mercaptoethanol) was added to the tube then incubated at 65 °C for 10 minutes. Next, 500 µl of chloroform/isoamyl alcohol (49:1 v/v) was added and the tube was vortexed at room temperature. The tube was then centrifuged for 15 minutes at 4 ^{0}C, 17,401 x *g*. This step was repeated two times and the supernatant was transferred to a new 2 ml microcentrifuge tube. Then, 500 µl of chloroform was added and the tube was vortexed at room temperature. The tube was then centrifuged for 15 minutes at 4 ^{0}C, 17,401 x *g* and the supernatant was transferred to a new 1.5 ml microcentrifuge tube. After that, 0.25 volumes of 10 M LiCl were added to the supernatant and incubated at -20 ^{0}C for overnight. Next, the tube was centrifuged for 15 minutes at 4 ^{0}C, 17,401 x *g*. The pellet was dissolved in 500 µl of TE buffer (10 mM Tris-Cl, pH 7.5 and 1 mM EDTA) then added with 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1 v/v, pH 6.7). The tube was vortexed at room temperature then centrifuged for 15 minutes at 4 ^{0}C, 17,401 x *g* and the supernatant was transferred to a new 2 ml microcentrifuge tube. Equal volume of chloroform/isoamyl alcohol (49:1 v/v) was added and vortexed at room temperature then centrifuged for 15 minutes at 4 ^{0}C, 17,401 x *g*. The supernatant was transferred to a new 1.5 ml microcentrifuge tube. Subsequently, 0.1 volume of 3M NaOAc, pH 5.2 and 2.5 volume of 100 % ethanol were added to the supernatant and incubated overnight at -20 ^{0}C. Next, the tube was centrifuged for 15 minutes at 4 ^{0}C, 17,401 x *g*. The pellet was washed by using 200 µl 70% ethanol and proceeded by centrifuging 17,401 x *g* for 15 minutes at 4 ^{0}C. Next, the supernatant was discarded and the tube was left to dry by inverting on a paper towel. The pellet was then dissolved in 30 µl of DEPC-treated water (Method modified from National Pingtung University of Science and Technology -Department of plant industry -Dr. Fu-chi chen http://agriculture.npust.edu.tw/npust_teacher125/intro/). Finally, extracted RNA was visualized by staining with 0.5 µg/ml of Ethidium bromide on a 1% agarose gel (The method was as shown in 3.2.18).

3.2.3.3 RNA extraction (Commercial kit)

This procedure used the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions and as follows. Fifty mg of sample was weighed and placed immediately in liquid nitrogen and ground thoroughly with a mortar and pestle. The powder was then decanted into an RNase-free, liquid nitrogen-cooled, microcentrifuge tube. Four hundred and fifty µl Buffer RLT (1 % β-mercaptoethanol added as directed by protocol) was added to the frozen tissue sample and the tubes were vortexed vigorously. The lysate was then applied directly into a QIAshredder spin column placed in a 2 ml collection tube and centrifuged for 2 minutes at 5,031 x *g*. The supernatant was then transferred to a new microcentrifuge tube without disturbing the cell-debris pellet at the bottom of the tube. A half volume of ethanol was added to the clear lysate and mixed by pipetting. The sample was then applied to an RNeasy mini column placed in a 2 ml collection tube. The tube was then centrifuged for 1 minute at 15,000 x *g* and the flow-through was discarded. Then, seven hundred µl Buffer RW1 was added to the RNeasy spin column and centrifuged for 1 minute at 8,000 x *g*. The flow-through was discarded. The RNeasy column was next transferred to a new collection tube. Later, 500 µl of Buffer RPE was pipetted into the RNeasy column and centrifuged for 1 minute at 10,000 x g. The flow-through was discarded again. Another 500 µl of Buffer RPE was added to the RNeasy column and centrifuged for 2 minutes at 10,000 x *g*. To elute, the RNeasy column was transferred to a new 1.5 ml collection tube and 30 µl of RNase free water was pipetted directly into column and centrifuged for 1 minute at 10,000 x *g*. Finally, extracted RNA was visualized by staining with 0.5 µg/ml of Ethidium bromide on a 1% agarose gel (The method was as shown in 3.2.18).

3.2.4 Deoxyribonuclease (DNase) treatment of extracted RNA

This procedure used the DNase I amplification grade (Invitrogen, United States) according to the manufacturer's instructions and as follows. The following reagents

were mixed in a 1.5 ml tube, on ice. The mixture was incubated at room temperature for 15 minutes. Then, 1 µl of 25 mM EDTA was added to the mixture and incubated at 65 ^{o}C for 15 minutes to heat inactivate the DNase I. Next, the mixture was replaced on ice for 1 minute before use for reverse transcription.

Table 3. 3: Reaction mixture set up for DNase I treatment.

Reaction Component	Volume, for 10µl of reaction mixture (µl)	Final concentration
RNA	variable	1 µg
10 X Reaction Buffer	1	1 X
DNase I (1 U/µl)	1	I U
DEPC-treated water	to 10 µl	

3.2.5 cDNA reverse transcription

Reverse transcription of RNA was carried out using a commercial kit, High capacity cDNA reverse transcription (Applied Biosystem, United States). One µg of total RNA was used for each 20 µl reaction. The composition of the reaction was as shown below. Subsequently, master mixes were placed on ice and mixed gently. Then, 10 µl of master mixes were pipetted into each 0.2 ml PCR tube. Next, 10 µl of each RNA sample was pipetted into each PCR tube and pipetted up and down to mix it. The tubes were centrifuged to spin down the contents and eliminate any air bubbles.

Table 3.4: Reaction mixture set up for reverse transcription.

Reaction Component	Volume, for 20µl of reaction mixture (µl)	Final concentration
10 X RT buffer	2	1 X
25 X dNTP mix (100 mM)	0.8	4 mM
10 X RT random primers	2	1 X
MultiScribe™ reverse transcriptase 50 U/µl	1	50 U
RNase inhibitor	1	40 U
nuclease free water	3.2	
Total RNA	10	1 µg

Table 3.5: Reverse transcription thermal cycle profile.

Step	Temperature (^{o}C)	Duration
1	25	10 min
2	37	120 min
3	85	5 min
4	4	∞

Next, the cDNA was spun down to eliminate any air bubbles. The cDNA products were stored at -20 ^{o}C for long term storage.

3.2.6 Rapid Amplification of cDNA Ends (RACE)

3.2.6.1 5’ Rapid Amplification of cDNA Ends (5’ RACE)

5’ RACE was carried out using a commercial kit, GeneRacer™ Kit (Invitrogen, United States). The following 10 µl dephosphorylation reaction was set up on ice in a 1.5 ml sterile microcentrifuge tube as shown in table 3.6 and mix gently by pipetting and vortexed briefly. The tubes were centrifuged to spin down the contents and incubate at 50 °C for 1 hour. Next, 90 µl DEPC water and 100 µl phenol:chloroform were added and vortex vigorously for 30 seconds (step 5). The tubes were centrifuged for 5 minutes at room temperature, 5,031 x *g*. Aqueous phase was transferred to a new 1.5 ml microcentrifuge tube then 2 µl 10 mg/ml mussel glycogen and 10 µl 3 M sodium acetate, pH 5.2 were added into the tubes and mixed well. Subsequently, 220 µl 95% ethanol was added and vortexed briefly. The tubes were frozen on dry ice for 10

minutes. Then, the tubes were centrifuged for 20 minutes at 4 °C, 5,031 x *g*. Supernatant was removed by pipetting. Five hundred µl 70% ethanol was added into the tubes, inverted several times and vortexed briefly. Next, the tubes were centrifuged for 2 minutes at 4 °C, 5,031 x *g*. The supernatant was discarded and the tube was left to dry by inverting on a paper towel. The pellet was then dissolved in 7 µl of DEPC-treated water (step 14). The following 10 µl decapping reaction was set up on ice in a 1.5 ml sterile microcentrifuge tube as shown in table 3.7 and mix gently by pipetting and vortexed briefly. The tubes were centrifuged to spin down the contents and incubated at 37 °C for 1 hour. Then, step 5 to 14 was repeated. Next, 7 µl of dephosphorylated, decapped RNA was added to the tube containing the pre-aliquoted, lyophilized GeneRacer™ RNA Oligo (0.25 µg) and pipetted up and down several times to mix and resuspend RNA Oligo. Then, the tubes were incubated at 65 °C for 5 minutes. The tubes were placed on ice for 2 minutes and centrifuged briefly. The following 10 µl ligation reaction was set up on ice in a 1.5 ml sterile microcentrifuge tube as shown in table 3.8 and mix gently by pipetting and vortexed briefly. The tubes were centrifuged to spin down the contents and incubate at 37 °C for 1 hour. Then, steps 5 to 13 were repeated. The pellet was then dissolved in 10 µl of DEPC-treated water. Subsequently, the reagents in table 3.9 were added into 10 µl of ligated RNA. The tubes were incubated at 65 °C for 5 minutes then chilled on ice for 2 minutes and centrifuged briefly. The reagents in table 3.10 were added to the 13 µl ligated RNA and primer mixture. The reagents were mixed well by pipetting gently up and down and then centrifuged briefly and incubated at 50 °C for 1 hour; follow by 70 °C for 15 minutes. The tubes were chilled on ice for 2 minutes and centrifuged briefly at 5,031 x *g*. Next, 1 µl of RNase H (2 U) was added to the reaction mix and incubated at 37 °C for 20 minutes. Finally, the cDNA was centrifuged briefly and used immediately for amplification or stored at -20 °C.

Table 3.6: Reaction mixture set up for dephosphorylating RNA.

Reaction Component	Sample RNA (µl)	Control RNA (µl)	Final concentration
RNA	variable	2	1 µg
10 X CIP Buffer	1	1	1 X
RNaseOut™ (40 U/µl)	1	1	40 U
CIP (10 U/µl)	1	1	10 U
DEPC water	to 10 µl	5	
Total volume	10	10	

Table 3.7: Reaction mixture set up for decapping RNA.

Reaction Component	Sample RNA (µl)	Final concentration
Dephosphorylated RNA	7	
10 X TAP Buffer	1	1 X
RNaseOut™ (40 U/µl)	1	40 U
TAP (0.5 U/µl)	1	0.5 U
Total volume	10	

Table 3.8: Reaction mixture set up for ligating the RNA oligo to decapped mRNA.

Reaction Component	Sample RNA (µl)	Final concentration
Dephosphorylated, decapped RNA	6	
10 X Ligase Buffer	1	1 X
10 mM ATP	1	1 mM
RNaseOut™ (40 U/µl)	1	40 U
T4 RNA ligase (5 U/µl)	1	5 U
Total volume	10	

Table 3.9: Reaction mixture set up 1 for reverse transcribing mRNA.

Reaction Component	Sample RNA (µl)	Final concentration
Ligated RNA	10	
GeneRacer™ Oligo dT Primer (50 µM)	1	2.5 µM
dNTP Mix (10 mM)	1	0.5 mM
Sterile, distilled water	1	
Total volume	13	

Table 3.10: Reaction mixture set up 2 for reverse transcribing mRNA.

Reaction Component	Sample RNA (µl)	Final concentration
5 X First Strand Buffer	4	1 x
0.1 M DTT	1	5 mM
RNaseOut (40 U/µl)	1	40 U
SuperScript™ III RT (200 U/µl)	1	200 U
Total volume	7	

3.2.6.2 3' Rapid Amplification of cDNA Ends (3' RACE)

Ten µl of total RNA was mixed with primer mix as shown in table 3.11. Then, the tubes were incubated at 65 °C for 5 minutes. The tubes were placed on ice for 2 minutes and centrifuged briefly. The reagents in table 3.10 were added to the 13 µl RNA and primer mixture. The reagents were mixed well by pipetting up and down gently and then centrifuged briefly and incubated at 50 °C for 1 hour; follow by 70 °C for 15 minutes. The tubes were chilled on ice for 2 minutes and centrifuged briefly. Next, 1 µl of RNase H (2 U) was added to the reaction mix and incubated at 37 °C for 20 minutes. Finally, the cDNA was centrifuged briefly and use immediately for amplification or stored at -20 °C.

Table 3.11: Reaction mixture set up for 3' reverse transcribing mRNA.

Reaction Component	Sample RNA (µl)	Final concentration
RNA	10	
GeneRacer™ Oligo dT Primer (50 µM)	1	2.5 µM
dNTP Mix (10 mM)	1	0.5 mM
Sterile, distilled water	1	
Total volume	13	

3.2.7 Polymerase chain reaction (PCR)

PCR was carried out using a commercial kit, i-Taq^{TM} DNA polymerase (Intron biotechnology, Korea). All solutions were gently vortexed and briefly centrifuged after thawing. After the mixture was added into the thin-walled 0.2 ml PCR tubes, the tubes were centrifuged to spin down the contents and eliminate any air bubbles. Samples were placed in Mastercycler Gradient DNA Thermocycler (Eppendorf, Germany) and PCR

was started. For every reaction, a negative control (without cDNA or DNA template) was prepared. Finally, PCR products were visualized by staining with 0.5 µg/ml of Ethidium bromide on a 1% agarose gel (The method was as shown in 3.2.18). Composition of each reaction was as shown below.

Table 3.12: Polymerase chain reaction mixture set up.

Reaction Component	Volume, for 20 µl of reaction mixture (µl)	Final concentration
10 x PCR buffer (100 mM Tris-HCI, pH 8.3, 500 mM KCI, 20 mM $MgCl_2$ and enhancer solution)	2	1 x
10 mM dNTP mixture (2.5 mM each)	2	1 mM
Forward primer (10 µM)	1	0.5 mM
Reverse primer (10 µM)	1	0.5 mM
Template	variable	1 ng plasmid DNA 100 ng genomic DNA 250 ng cDNA
Taq DNA polymerase (5 U/µl)	0.125	0.625 U
Sterile distilled water	to 20 µl	

Table 3.13: Polymerase chain reaction thermal cycle profile.

Step	PCR cycle	Temperature (^oC)	Duration
1	Initial denaturation	95	3 min
2	Denaturation	95	30 s
3	Annealing	variable	30 s
4	Extension	72	45 s
5	Repeat	step 2 to 4 for	34 cycles
6	Final extension	72	5 min
7	Hold	4	∞

3.2.8 Gradient Polymerase Chain Reaction

Gradient PCR was carried out by changing the annealing temperature of normal PCR to (Tm – 5 ^oC) ± 10 ^oC using Mastercycler Gradient DNA Thermocycler (Eppendorf, Germany).

3.2.9 Quantitative real time PCR

Quantitative real time PCR was carried out using Power SYBR® Green PCR Master Mix kit (Applied Biosystems, United States). The PCR Master Mix was prepared as shown in table 3.14. The PCR Master mixes were prepared using both β-actin and Mixta qPCR primers for the endogenous control wells as well as the sample wells. The master mix containing β-actin primers was added into the endogenous control wells while the master mix containing the Mixta qPCR primers were added into the sample wells. cDNA and nuclease free water was then added to the specific wells. The optical 96 well plate was then loaded into the Applied Biosystem 7500 Real-Time PCR system and the SDS 1.3.1 (Sequence Detection Software) was used to create a relative quantification (ddCt) plate. The run was then started. After the running of the program, the SDS software was again used to generate a dissociation curve and later a relative quantification (ddCt) study was created to analyze the results. The SDS software uses the equation $2^{-\Delta\Delta CT}$ to calculate the expression level of the *DhMYB Mixta-like* relative to the *β-actin* endogenous control.

Table 3.14: The reaction components for quantitative real time PCR

Reaction Component	Volume, for 20 µl of reaction mixture (µl)	Final concentration
2 X SYBR® Green Power Master Mix	10	1 X
Forward primer (10 µM)	1	0.5 mM
Reverse primer (10 µM)	1	0.5 mM
cDNA	2	10-100ng
Nuclease free water	6	

3.2.10 DNA purification

DNA purification was carried out using a commercial kit, QIAquick Gel Extraction Kit (Qiagen, Germany). DNA fragment was excised from the agarose gel with a clean, sharp scalpel. Then, the gel slice was weighed in a 2.0 ml microcentrifuge

tube. Three volumes of Buffer QG to 1 volume of gel were added. The mixture was incubated at 50 °C for 10 minutes. The tube was vortexed every 3 minutes during the incubation. Subsequently, 1 gel volume of isopropanol was added to the sample and mixed. The sample was applied to the QIAquick column, and centrifuged for 1 minute at 10,000 x *g*. Later, the flow-through was discarded. Five hundred ml of Buffer QG was added to QIAquick column and centrifuged for 1 minute at 10,000 x *g*. Next, 750 µl of Buffer PE was added to QIAquick column and centrifuged for 1 minute at 10,000 x *g*. The flow-through was discarded then the QIAquick column was centrifuged for an additional 1 minute at 10,000 x *g*. The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube. Fifty µl of elution buffer (10 mM Tris·Cl, pH 8.5) was added to the center of the QIAquick membrane and the column centrifuged for 1 minute at 12,000 x *g* to elute DNA. Then, DNA was visualized by staining with 0.5 µg/ml of Ethidium bromide on a 1% agarose gel (The method was as shown in 3.2.18) prior proceed to cloning step.

3.2.11.1 Preparation of competent *E.coli* HT115 (DE3) cells

Single colonies of *E.coli* HT115 (DE3) cells (F-mcrA mcrB IN (rrnD-rrnE) 1, rnc14::Tn10 (DE3 lysogen:lacUV5 promoter-T7 polymerase, tetR) (Dasgupta *et al*., 1998; Takiff *et al*., 1989) was inoculated into 5 ml of LB/Tet (12.5µg/ml) broth and grown overnight at 37 °C, at 220 rpm. Next, 100 µl of the overnight inoculum was diluted 100 times in 10 ml LB broth containing tetracycline (12.5µg/ml) and incubated at 37 °C, 220 rpm until the cells reached the exponential phase (OD_{600}=0.4). The culture was then centrifuged at 4,528 x *g*, 4 °C for 10 minutes and the pellet was resuspended in half of the original volume of ice cold 50 mM $CaCI_2$ and incubated on ice for 30 minutes followed by centrifugation at 4,528 x *g*, 4 °C for 10 minutes. The pellet was then resuspended in 0.1 of the original volume of ice cold 50 mM $CaCI_2$. The mixture

was aliquoted into 1.5 ml microcentrifuge tubes and stored in 10 % glycerol at -80°C or used immediately.

3.2.11.2 Preparation of competent *E. coli* XL1-Blue cells

Single colonies of *E.coli* XL1-Blue cells (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacIqZΔM15* Tn*10* (Tet^r)] was inoculated in 5 ml of LB broth and grown overnight at 37 °C, at 220 rpm. Next, 100 µl of the overnight inoculum was diluted 100 times in 10 ml LB broth and incubated at 37 °C, 220 rpm until the cells reached the exponential phase (OD_{600}=0.4). The culture was kept on ice for 10 minutes. Next, the culture was centrifuged at 1,500 x *g*, 4 °C for 10 minutes. The pellet was then resuspended in 0.1 of the original volume of culture media (LB with 10 mM $MgSO_4$ and 0.2 % glucose) on ice. Subsequently, 0.5 of the original volume of storage solution (36 % glycerin, 12 % PEG MW 6000, 12 mM $MgSO_4$ in LB, pH7.0) was added in the mixture. Finally, the mixture was aliquoted into 1.5 ml microcentrifuge tubes and used immediately or stored in 15 % glycerol at -80 °C.

3.2.12 Ligation

3.2.12.1 TA cloning

Ligation was carried out using a commercial vector, pGEM®-T Easy Vector Systems (Promega, United States). pGEM®-T Easy cyclized at the EcoRI site and used for the construction of different vectors. Ligation reaction mixture of samples, positive control and background control were prepared as shown in table 3.15. Then, the reactions were incubated overnight at 4 °C and used directly for bacterial transformation.

Table 3.15: Ligation reaction set up.

Reaction Component	Standard Reaction(µl)	Positive Control(µl)	Background Control(µl)	Final concentration
2 X Rapid Ligation Buffer	5	5	5	1 X
pGEM®-T Easy Vector (50 ng/µl)	1	1	1	50 ng
PCR product	3	-	-	25 – 50 ng
Control Insert DNA	-	2	-	8 ng
T4 DNA Ligase (3 Weiss units/µl)	1	1	1	3 Weiss units
nuclease-free water to a final volume of	10	10	10	

3.2.12.2 Blunt end cloning

Ligation was carried out using a commercial vector, CloneJET™ PCR Cloning Kit (Fermentas, Europe). pJET1.2/blunt is a linearized cloning vector, which accepts inserts from 6 bp to 10 kb. The reaction component was set up on ice as shown in table 3.16. The mixture was vortexed briefly and centrifuged for 5 seconds. Next, the ligation mixture was incubated at room temperature for 5 minutes and used directly for bacterial transformation.

Table 3.16: Blunting reaction set up.

Reaction Component	Volume (µl)	Final concentration
2 X Reaction Buffer	10	1 X
PCR product	2	50 – 100 ng
pJET1.2/blunt Cloning Vector (50 ng/µl)	1	50 ng
T4 DNA Ligase (5 U/µl)	1	5U
Water, nuclease-free	6	
Total volume	20	

3.2.13 Transformation

3.2.13.1 Transformation of *E. coli* HT115 (DE3) cells

One to one hundred ng of plasmid was added to 200 µl of competent HT115 cells in a polypropylene tube and incubated on ice for 30 minutes. The tube was then incubated in a 37 °C water bath for 1 minute followed by incubation on ice for 2

minutes. One ml of SOC media (20 g bacto tryptone, 5 g bacto yeast extract, 2 ml of 5 M NaCI, 2.5 ml of 1 M KCI, 10 ml of 1 M $MgCI_2$, 10 ml of 1 M $MgSO_4$ and 20 ml of 1 M glucose in 1 L H_2O) was added to the tube and incubated for 37 °C with 220 rpm shaking for 1 hour. The culture was then plated on LB agar/ampicillin (100 μg/ml) /tetracycline (12.5 μg/ml) at volumes of 10 μl, 100 μl and 200 μl. The plates were then incubated at 37 °C overnight for at least 36 hours to allow colony formation, as HT115 are slow growing cells.

3.2.13.2 Transformation of *E. coli* XL1-Blue cells

Fifty ng of plasmid was added to 100 μL of *E. coli* XL-1 Blue competent cells in a polypropylene tube and incubated on ice for 30 minutes. The tube was then incubated in a 42 °C water bath for 1 minute followed by incubation on ice for 2 minutes. One ml of culture medium (LB with 10 mM $MgSO_4$ and 0.2 % glucose) was added to the tube and incubated for 37 °C with shaking 220 rpm for 1 hour. The culture was then plated on LB agar/ampicillin (100 μg/ml)/x-gal (80 μg/ml)/0.5 mM IPTG at volumes of 10 μl, 100 μl and 200 μl. The plates were then incubated at 37 °C overnight for 18 hours to allow colony formation.

3.2.14 Plasmid preparation

This procedure used the Wizard® Plus SV Minipreps DNA Purification System (Promega, United States) according to the manufacturer's instructions and as follows. Three ml of overnight bacterial culture was harvested by centrifugation for 5 minutes at 10,000 x *g* in a tabletop centrifuge. The supernatant was poured off and the inverted tube was blotted on a paper towel to remove excess media. Then, 250 μl of cell resuspension solution was added and the cell pellet was completely resuspended by vortexing. Next, 250 μl of cell lysis solution was added and mix by inverting the tube 4

times. The mixture was incubated for 3 minutes until the cell suspension clears. Next, 10 µl of alkaline protease solution was added and mixed by inverting the tube 4 times. The mixture was incubated for 5 minutes at room temperature. Subsequently, 350µl of neutralization solution was added and immediately mixed by inverting the tube 4 times. The bacterial lysate was centrifuged at 14,000 × *g* for 10 minutes at room temperature. The cleared lysate was added to the prepared spin column by decanting. The supernatant was centrifuged at 14,000 × *g* for 1 minute at room temperature. The flow through from the collection tube was discarded. Then, 750 µl of column wash solution was added to the spin column and centrifuged at 14,000 × g for 1 minute at room temperature. Again, the flow through was discarded. To elute, 50 µl of nuclease-free water was added to the spin column and centrifuged at 14,000 × g for 1 minute at room temperature. Finally, DNA was visualized by staining with 0.5 µg/ml of Ethidium bromide on a 1% agarose gel (The method was as shown in 3.2.18) and then proceed to DNA sequencing.

3.2.15 Restriction enzyme digestion

This procedure used the restriction enzyme (New England Biolabs, United States) according to the manufacturer's instructions and as follows. The DNA samples were subjected to restriction enzyme digestion by incubating the reaction mixture at 37 °C for 2 hours. Then, the samples were incubated at 65 °C for 20 minutes to inactivate the enzyme.

Table 3.17: Digestion reaction set up.

Reaction Component	Volume (µl)	Final concentration
10 X NEBuffer 3	5	1 X
100 X BSA	0.5	1 X
Not I (10,000 U/ml)	0.5	5 U
Nco I (10,000 U/ml)	0.5	5 U
DNA	variable	1 µg
Water, nuclease-free	to 50 µl	
Total volume	50	

3.2.16 Sequencing

Purified DNA was sent to First Base Laboratories Sdn. Bhd. for sequencing. Sanger sequencing technique was carried out.

3.2.17 Bacterial expression of dsRNA

3.2.17.1 Induction of bacteria expression of candidate RNA

E. coli HT115 (DE3) bacteria containing the plasmids harboring the desired constructs were spread onto LB/Amp (100 µg/ml)/Tet (12.5 µg/ml) agar plates separately and grown overnight at 37 ^{0}C. Single colonies was inoculated into LB broth containing Tet (12.5 µg/ml) and Amp (100 µg/ml) and grown overnight with 220 rpm shaking at 37 °C. Each culture was then diluted 100 fold in 2 X YT broth (16 g tryptone, 10 g yeast extract and 5 g sodium chloride in 1 L H_20) with Tet (12.5 µg/ml) and Amp (100 µg/ml) antibiotics and allowed to grow to OD_{600} = 0.7. The T7 RNA polymerase gene in the HT115 (DE3) was induced by addition of 0.4 mM IPTG and the culture incubated with 220 rpm shaking for 2 hours at 37 °C. Then, IPTG-induced *E. coli* HT115 cells (100 ml) were pelleted by centrifuging at 4,000 x *g* for 10 minutes and resuspended in 200 µl resuspension buffer (10 mM EDTA, 50 mM Tris Base, pH 8.0). Then, 200 µl of lysozyme (1 mg/ml) was added to the mixture and incubated at room temperature for 5 minutes. The mixture was vortexed for few seconds in 2 minutes interval. Cells were pelleted again by centrifuging at 1,4000 x *g* for 5 minutes,

supernatant was removed from the cell debris. Accumulation of dsRNA was confirmed by using RNAse/DNase digestion. This procedure was used MEGAscript® RNAi Kit (Invitrogen, United States) according to the manufacturer's instructions and as follows. The reaction components were set up on ice as shown in table 3.18 and incubated at 37 °C for 1 hour. Then, the purification to remove proteins, free nucleotides, and nucleic acid degradation products from the dsRNA was carried out by setting up reaction components as shown in table 3.19. Next, 500 µl dsRNA binding mixture was place in the

Filter Cartridge and centrifuged at 1,4000 x *g* for 2 minutes. The flow-through was discarded and the Filter Cartridge was replaced in the Collection Tube. To wash the Filter Cartridge, 500 µL of 2 X Wash Solution was pipetted onto the filter in the Filter Cartridge and centrifuged at 1,4000 x *g* for 2 minutes. The flow-through was discarded and the Filter Cartridge was replaced in the Collection Tube. Then, the washing step was repeated one more time. After discarding the Wash Solution, the Filter Cartridge was centrifuged at 1,4000 x *g* for 1 minute to remove the last traces of liquid. The Filter Cartridge was transferred to a fresh Collection Tube. Next, 100 µL of preheated Elution Solution was added to the filter in the Filter Cartridge and centrifuged for 2 minutes at 1,4000 x *g*.

Table 3.18: Reaction set up of RNase and DNase treatment.

Reaction Component	Volume (µl)	Final concentration
10 X Digestion Buffer	5	1 X
DNase I (1U/µl)	2	2 U
RNase (1U/µl)	2	2 U
dsRNA (1µg/ul)	2	2 µg
Nuclease-free water	39	
Total	50	

Table 3.19: Reaction set up of dsRNA purification.

Reaction Component	Volume (µl)	Final concentration
10 X Binding Buffer	50	1 x
dsRNA (total from table 3.18)	50	
Nuclease-free water	150	
100 % etanol	250	
Total	500	

3.2.17.2 Large scale preparation of crude bacterial extracts

IPTG-induced *E. coli* HT115 cells (200 ml) were pelleted by centrifuging at 4,000 x *g* for 10 minutes and resuspended in 500 µl resuspension buffer (10 mM EDTA, 50 mM Tris Base, pH 8.0). Then, 500 µl of lysozyme (1 mg/ml) was added to the mixture and incubated at room temperature for 5 minutes. The mixture was vortexed for few seconds in 2 minutes interval. Cells were pelleted again by centrifuging at 1,4000 x *g* for 5 minutes, supernatant was removed from the cell debris and stored in -80 ^{0}C (modified from Timmons *et al*., 2001).

3.2.18 dsRNA inoculation

Crude bacterial extracts containing *DhMYB Mixta-like* dsRNA and empty vector L4440 dsRNA were inoculated onto three orchid plants at bud length 0-5 mm stage respectively. Mechanical inoculation of dsRNA was carried out by gently rubbing 100 µg of crude bacterial extracts onto the uppest leaf by 5 days interval using a latex-gloved finger. The plants were then left for 20 minutes before rinsing of any debris under slow running tap water. The plants then were allowed to grow at 25 ºC with 16 hours of light and 8 hours of dark photoperiod cycle.

3.2.19 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out to check the quality of the nucleic acid. First of all, 1 % of agarose powder was weighed and added into 1 X TBE buffer **(**Tris base, 0.5 M EDTA,pH 8.0 and Boric Acid) in the conical flask and swirled to mix.

The mixture was placed into a microwave oven and microwaved until the mixture was clear. Approximately 1.5 µl of ethidium bromide (10 mg/ml) was added and mixed. The mixture was then poured into the gel mould and any bubbles were removed. The gel was left to set for 25 minutes. Then, 2 µl of a 100 bp DNA ladder was loaded into the well and 5 µl of the samples were individually mixed with 1 µl of 6 x loading dye and then loaded into the wells. The gel was run for 30 minutes at 120 V. The gel was then visualized under ultraviolet light and a gel picture was taken by using Alphaimager^{TM} 2200 programme (International Equipment Trading Ltd., United States).

3.2.20 Scanning electron microscopy

Dendrobium hybrida lip, petal and sepal were cut into small pieces and soaked in primary fixative, 2% glutaraldehyde in distilled water at 4 ^{0}C for overnight followed by secondary fixative, 2% aqueous osmium tetroxide in distilled water at 4 ^{0}C for overnight. Then, the samples were washed with distilled water two times for 15 minutes. Subsequently, samples were dehydrated through ethanol series (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%; one time incubation for each dehydration except 100% ethanol with two times incubation) for 15 minutes. Next, samples were infiltrated through ethanol and acetone mixture (3:1, 1:1 and 1:3 of ethanol ratio acetone) for 15 minutes. After that, the samples were incubated in acetone for 1 hour, then dehydrated samples were subjected to critical point drying at 31.5 ^{0}C, 1,100 psi using a CPD 7501 instrument (Poloran, United Kingdom). Dried samples were mounted on specimen stubs and dried overnight in vacuum desiccators then were coated with gold. The gold coated samples were examined using a JSM 6400 scanning electron microscope (SEM) (Jeol Ltd., Japan). Cell shape observation was made from SEM micrographs at 100, 400 and 500 times magnification.

Fresh *Dendrobium hybrida* lip, petal and sepal were mounted on specimen stubs and examined using a Leo 1455 VP-SEM (Angstrom Scientific Inc., United States). Cell shape observation was made from SEM micrographs at 400 times magnification.

3.2.21 Phylogenetic study

Thirty-seven isolated putative partial protein sequences were aligned with DwMYB1 (accession no.: AF485892.1) using EMBL-EBI ClustalW2 (http://www.ebi.ac.uk/ Tools/msa/clustalw2/). Next, phylogenetic analysis was performed using IT3F: An Interspecies Transcription Factor Function Finder for Plants (http://jicbio.bbsrc.ac.uk/IT3F) and MEGA 4 software.

3.2.22 Statictical analysis

Relative quantification of *DhMYB* cDNA at different bud stages (0 - 3.5 cm) were analysed by ANOVA one tailed test whereas relative quantification of *DhMYB* cDNA between untreated control and treated bud stages (2 - 3.5 cm) were analysed by ANOVA two tailed test using statistica 10 software.

Chapter 4.0 Results

4.1 Isolation and characterization of *MYB* genes in orchid

4.1.1 Orchid DNA quality

The quality and amount of genomic DNA isolated from leaves of *Dendrobium hybrida* varied, as shown in table 4.1 and figure 4.1. DNA samples with an absorbance at 260 nm/ absorbance at 280 nm ratio between 1.65 and 1.85 (figure 4.1 lane 1, 2, 3 and 4) were selected as templates for primer optimization.

Table 4.1: Quality of Dendrobium hybrida genomic DNA

DNA	Concentration (ng/ µl)	OD: 260/280
1	262.3	1.76
2	125.1	1.70
3	131.7	1.80
4	150.4	1.78

4.1.2 Selection and optimization of *MYB* gene primers

Genomic DNA was used to optimise annealing temperatures for degenerate primer pairs Dh1-Myb and Dh2-Myb by gradient PCR. For degenerate primer pair Dh1-Myb, annealing temperatures of 48 ^{0}C and 51.2 ^{0}C gave three PCR products of approximately 400 bp, 450 bp and 650 bp in size (figure 4.2A). Only an annealing temperature of 58.8 ^{0}C gave a specific band at approximately 650bp and hence was selected for further use. Degenerate primer pair Dh2-Myb annealing temperatures of 47 ^{0}C to 63 ^{0}C gave three PCR products of approximately 380 bp, 600 bp and 900 bp in size whereas an annealing temperature of 52.5 ^{0}C produced the sharpest bands thus was selected for further use (figure 4.2B).

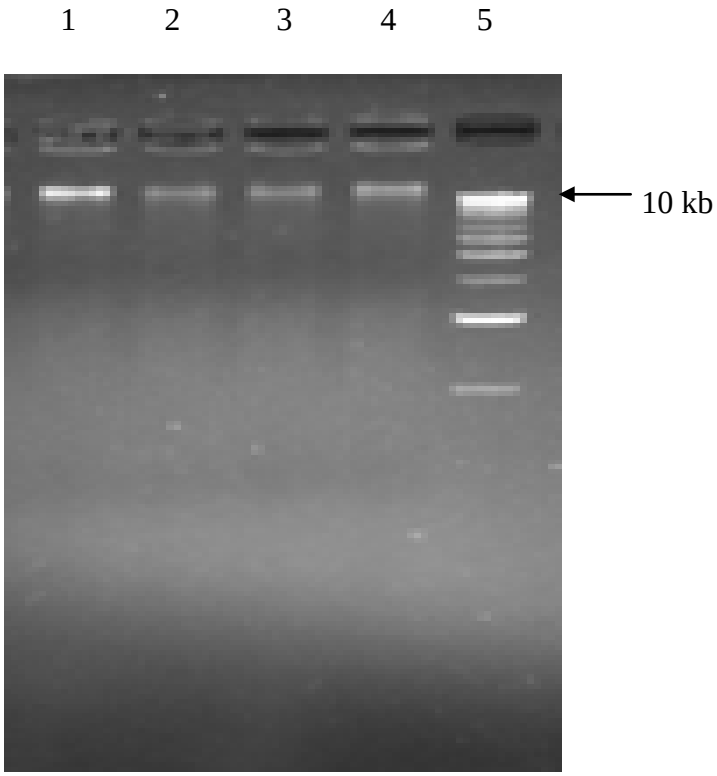


Figure 4.1: Agarose gel analysis of genomic DNA isolated from Dendrobium hybrida leaves. Lane 1 to lane 4: genomic DNA of *Dendrobium hybrida* leaves and lane 5: Fermentas 1kb DNA marker.

4.1.3 Orchid RNA quality

High quality RNA was obtained for the different stages of floral bud ranging from 0 to 3.5 cm, sepal, petal, lip and leaf, with two distinct bands observed and the 28S band more intense than the 18S band as shown in figure 4.3 (A and B). RNA samples with an absorbance at 260 nm/ absorbance at 280 nm ratio between 1.9 and 2.3 were reverse transcribed to cDNA and subjected to amplification. Quality of *Dendrobium hybrida* total RNA sample is shown in table 4.2.

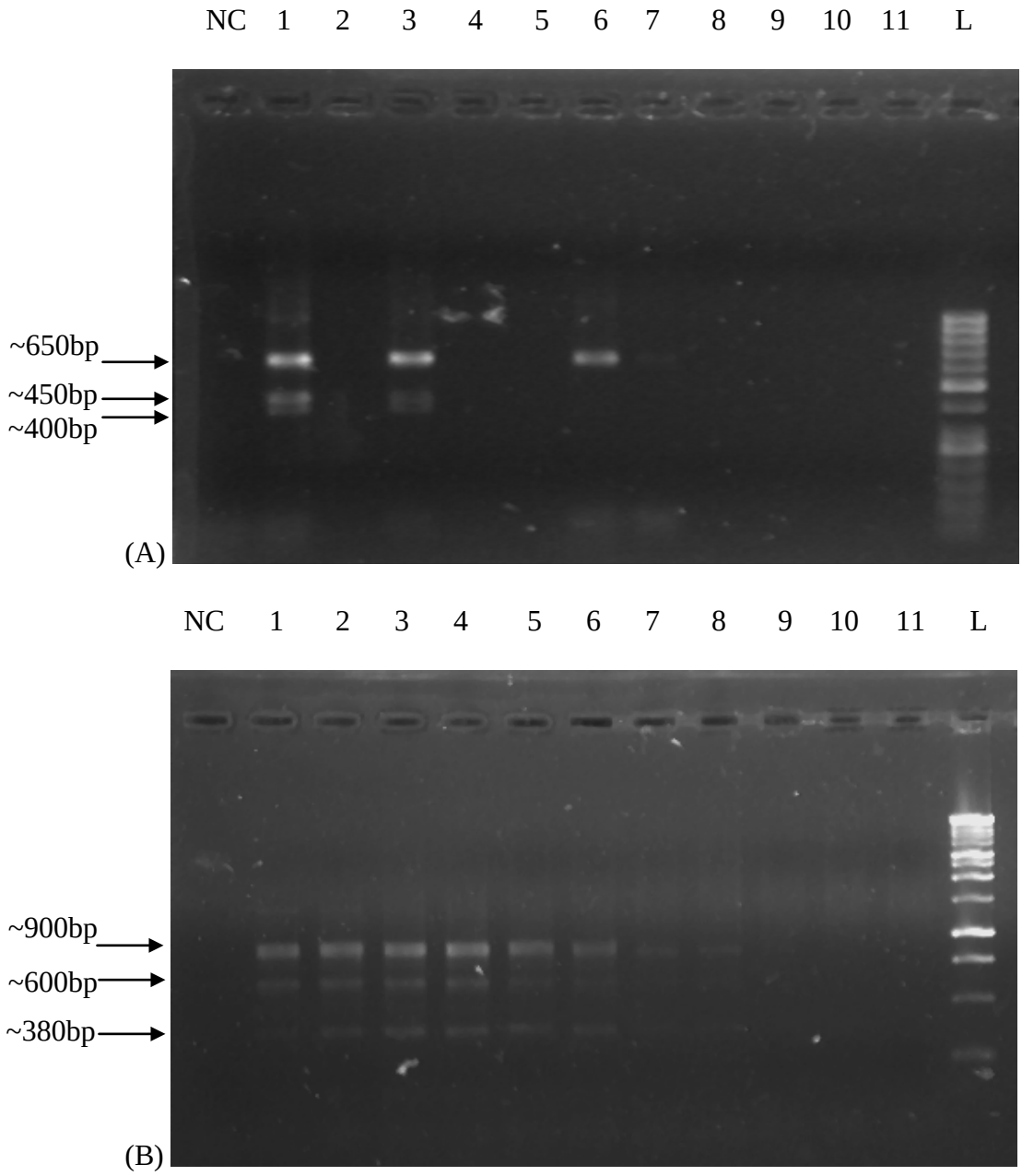


Figure 4.2: The effect of annealing temperature on PCR with degenerate primer pairs Dh1-Myb and Dh2-Myb. (A). Agarose gel of products from gradient PCR using Dh1-Myb primer pair; lane 1: annealing temperature 48 ^{0}C; lane 2: 49.4 ^{0}C; lane 3: 51.2 ^{0}C; lane 4: 53.5 ^{0}C; lane 5: 56.1 ^{0}C; lane 6: 58.8 ^{0}C; lane 7: 61.5 ^{0}C; lane 8: 64 ^{0}C; lane 9: 66.1 ^{0}C; lane 10: 67.7 ^{0}C; lane 11: 68.5 ^{0}C; lane L: Fermentas 100bp DNA marker. (B). Agarose gel of products from gradient PCR using Dh2-Myb primer pair; lane 1: annealing temperature 47 ^{0}C; lane 2: 48.4 ^{0}C; lane 3: 50.2 ^{0}C; lane 4: 52.5 ^{0}C; lane 5: 55.1 ^{0}C; lane 6: 57.8 ^{0}C; lane 7: 60.5 ^{0}C; lane 8: 63 ^{0}C; lane 9: 65.1 ^{0}C; lane 10: 66.7 ^{0}C; lane 11: 67.5 ^{0}C; lane L: Fermentas 1kb DNA marker. For all gels, NC: negative control of amplification.

Table 4.2: Quality of Dendrobium hybrida total RNA samples

RNA Sample	Concentration (ng/ µl)	OD: 260/280
0-0.5 cm bud	78.8	2.18
0.5-1.0 cm bud	96.3	2.18
1.0-1.5 cm bud	98.4	2.17
1.5-2.0 cm bud	106.4	2.19
2.0-2.5 cm bud	114.5	2.19
2.5-3.0 cm bud	90.4	2.23
>3.0 cm bud	84.3	2.22
sepal	94.8	2.20
petal	90.9	2.21
lip	109.5	2.20
leaf	92.2	2.21

4.1.4 cDNA synthesis

cDNA synthesis efficiency of all cDNAs was tested by PCR using a *β-actin* housekeeping gene primer pair. All PCR products gave a single band at 150 bp as shown in figure 4.4.

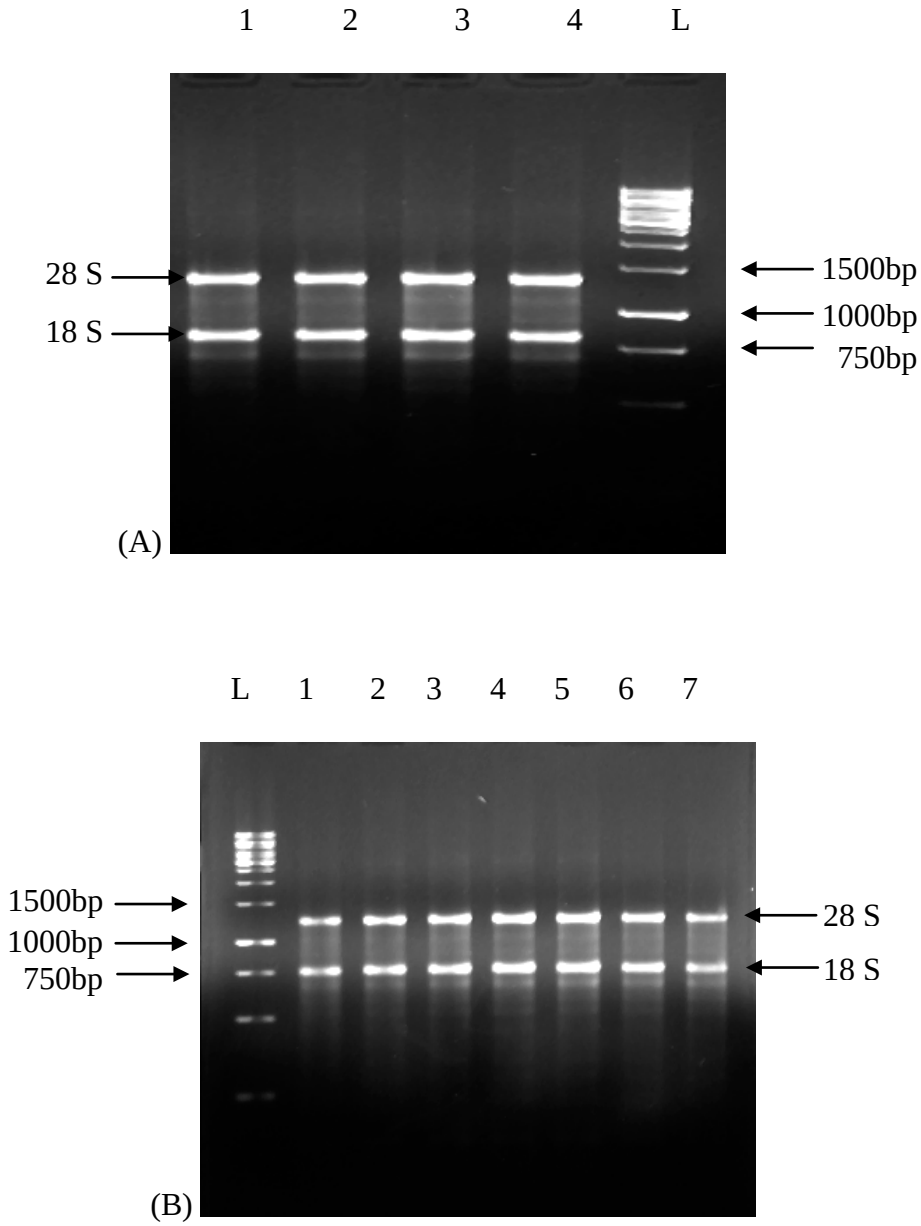


Figure 4.3: Agarose gel analysis of total RNA extracted from various parts of Dendrobium hybrid. (A). Total RNA extracted from different bud size stages; lane 1: 0-0.5 cm; lane 2: 0.5-1.0 cm; lane 3: 1.0-1.5 cm; lane 4: 1.5-2.0 cm. (B). Total RNA of different tissues; lane 1: 2.0-2.5 cm; lane 2: 2.5-3.0 cm; lane 3: more than 3.0 cm; lane 4: sepal; lane 5: petal; lane 6: lip; lane 7: leaf. For all gels, L: Fermentas 1kb DNA marker.

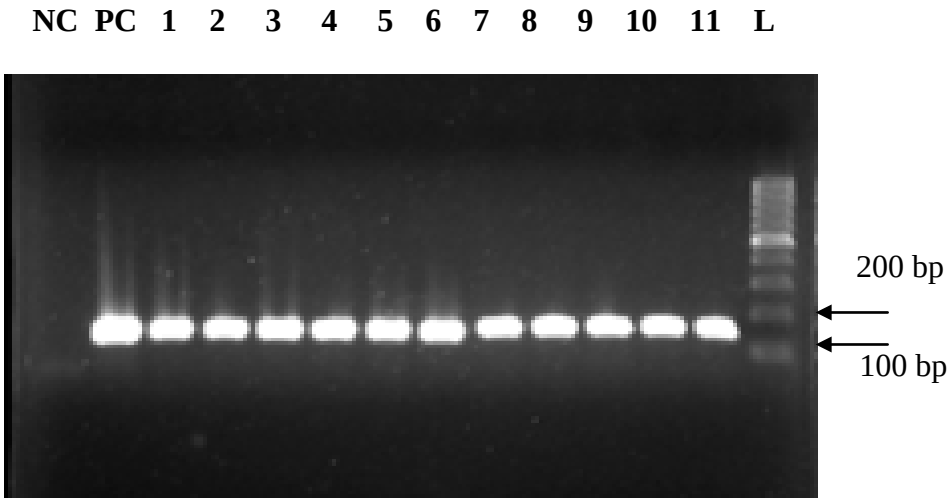


Figure 4.4: Agarose gel analysis of RT-PCR products amplified by β-actin housekeeping gene primer pair; lane 1: bud size 0-0.5 cm; lane 2: 0.5-1.0 cm; lane 3: 1.0-1.5 cm; lane 4: 1.5-2.0 cm; lane 5: 2.0-2.5 cm; lane 6: 2.5-3.0 cm; lane 7: more than 3.0 cm; lane 8: sepal; lane 9: petal; lane 10: lip; lane 11: leaf; NC: negative control of amplification; PC: positive control of amplification; L: Fermentas 100 bp DNA marker.

4.1.5 *MYB* cDNA amplification

High quality RNAs were reverse transcribed to cDNA and subjected to amplification. Amplification of cDNA from the smallest-sized floral buds (0-0.5 cm) using the Dh1-Myb primer pair gave four products of approximately 300 bp, 400 bp, 600 bp and 700 bp in size whereas amplification of petal, medium-sized floral bud (1.0-1.5 cm), mature floral bud (3.0-3.5 cm), sepal and leaf cDNA gave a single product of approximately 300bp as shown in figure 4.5 A and B. No PCR product was observed for lip cDNA as shown in figure 4.5 A. Amplification of floral buds and leaf cDNA using the Dh2-Myb primer pair gave a sharp band at approximately 300bp whereas amplification of lip and petal cDNA gave a faint band at approximately 300bp whilst no PCR product was observed for sepal cDNA as shown in figure 4.5 C and D.

4.1.6 *MYB* cDNA cloning

A single PCR product for each tissue of *Dendrobium hybrida* (i.e. leaf, smallest-sized floral bud (0-0.5 cm), medium floral bud (1-1.5 cm), mature floral bud (3-3.5 cm), lip and petal) and each primer pair (i.e. Dh1-Myb and Dh2-Myb) was column purified. Each was then ligated into TA vector (pGEM®-T Easy Vector) followed by transformation into *E. coli* XL1-Blue strain. Twenty white colonies per transformation were selected for colony PCR and 10 insert containing plasmids were sequenced for each. Typical PCR products (~370 bp) from colony selection for different transformations are shown in figures 4.6 and 4.7.

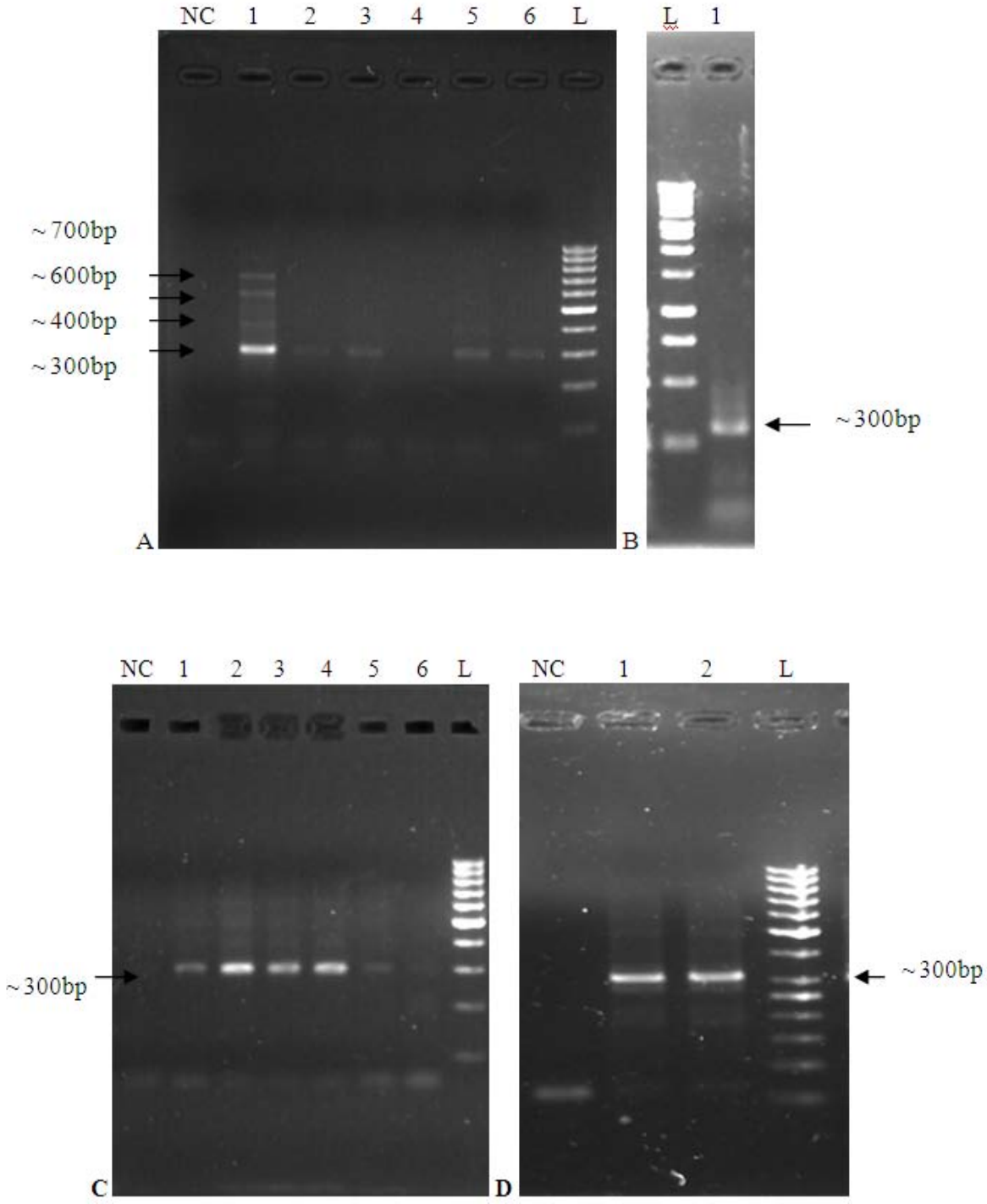


Figure 4.5: Agarose gel analysis of RT-PCR products amplified by Dh1-Myb and Dh2-Myb primer pairs. (A). Amplification of MYB cDNA using the Dh1-Myb degenerate primer pair on different tissues; lane 1: smallest-sized floral bud (0-0.5 cm); lane 2: medium-sized floral bud (1.0-1.5 cm); lane 3: mature floral bud (3.0-3.5 cm); lane 4: lip; lane 5: petal; lane 6: sepal; lane L: Fermentas 100bp DNA marker. (B). Dh1-Myb degenerate primer pair using leaf cDNA as template; lane 1: leaf; lane L: Fermentas 1kb DNA marker. (C). Amplification of *MYB* cDNA using Dh2-Myb degenerate primer pair and cDNA from different tissues; lane 1: lip; lane 2: smallest-sized floral bud (0-0.5 cm); lane 3: medium-sized floral bud (1.0-1.5 cm); lane 4: mature floral bud (3.0-3.5 cm); lane 5**:** petal; lane 6: sepal; lane L: Fermentas 100bp

DNA marker. (D). Amplification using Dh2-Myb degenerate primer pair and leaf cDNA as template; lane 1 and lane 2: leaf; lane L: Fermentas 50bp DNA marker. For all gels, NC: negative control of amplification.

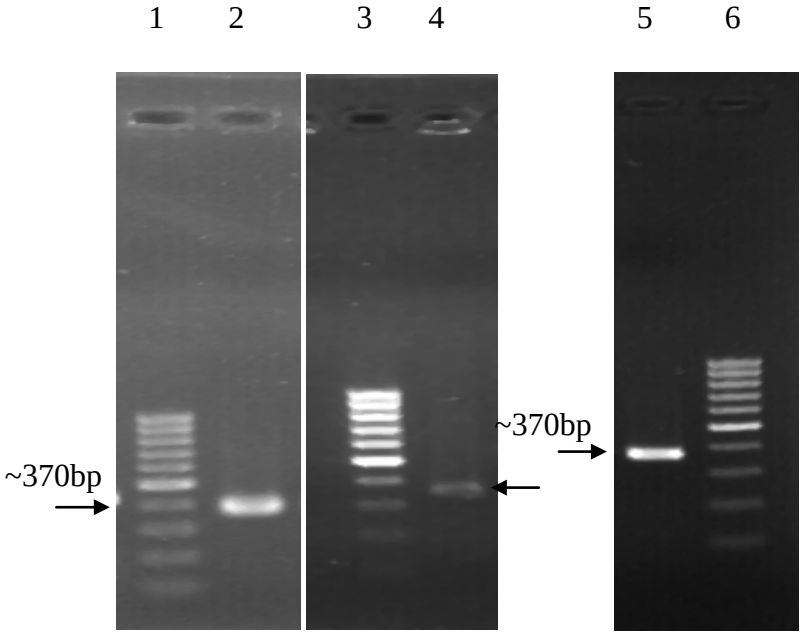


Figure 4.6: Colony PCR to select clones carrying Dh1 MYB cDNA. T7 promoter forward primer and Dh1-Myb reverse primer were used in colony PCR to screen colonies that harboured Dh1-Myb cDNA from different tissues, followed by agarose gel electrophoresis; lane 2: leaf; lane 4: smallest-sized floral bud (0-0.5 cm); lane 5: petal; lane 1, 3 and 6: Fermentas 100 bp DNA marker.

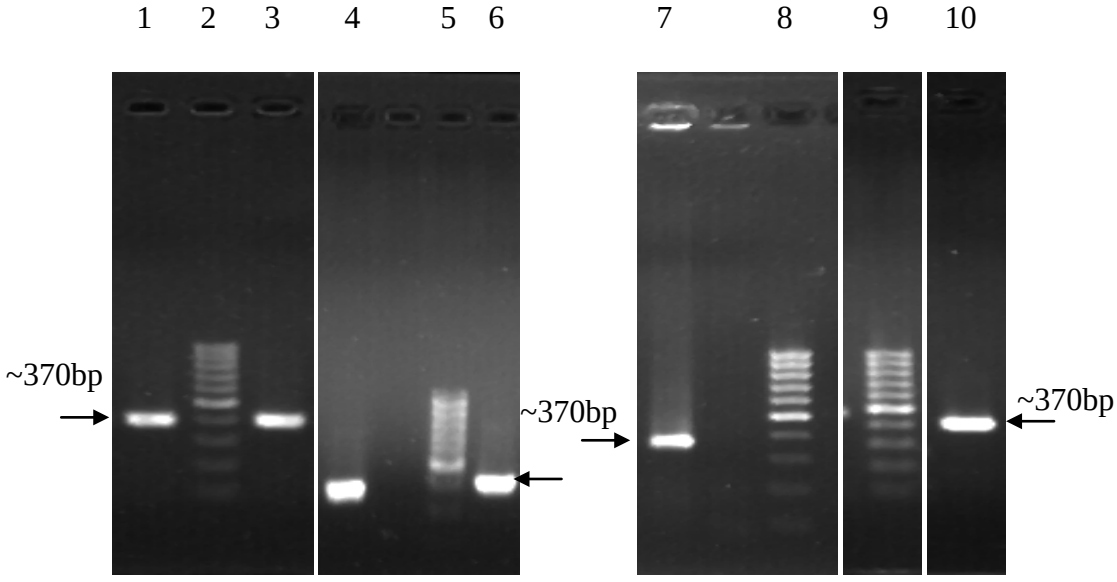


Figure 4.7: Colony PCR to select clones carrying Dh2 MYB cDNA. T7 promoter forward primer and Dh2-Myb reverse primer were used in colony PCR to screen colonies that harbour Dh2 *MYB* cDNA from different tissues, followed by agarose gel electrophoresis; lane 1: medium-sized floral bud (1-1.5 cm); lane 3: mature floral bud (3-3.5 cm); lane 4: lip; lane 6: smallest-sized floral bud (0-0.5 cm); lane 7: leaf; lane 10: petal; lane 2, 5, 8 and 9: Fermentas 100 bp DNA marker.

4.1.7 Alignment between MYB proteins of Dendrobium hybrida and Dendrobium sp. XMW-2002-21 MYB1 protein

Dh1-Myb and Dh2-Myb primers that were designed from the conserved region of the N-terminal DNA binding domain of MYB proteins from *Petunia hybrida*, *Arabidopsis thaliana* and *Antirrhinum majus* successfully amplified PCR products from *Dendrobium hybrida*. Nine and twenty eight unique sequences were identified using Dh1-Myb and Dh2-Myb primer pairs respectively as shown in table 4.3. The 37 sequences encoding MYB proteins in *Dendrobium hybrida* were categorized as belonging to the R2R3-MYB gene family due to the presence and characteristics of two repeat regions, which are most similar to R2 and R3 of the DwMYB1: *Dendrobium sp. XMW-2002-1* MYB 1 (GenBank accession number: AAO49410). These two repeat regions are constituted by two imperfect repeats of 50 amino acids. The R2 repeat has three regularly spaced tryptophan residues and the R3 repeat includes two tryptophan and one phenylalanine residue which encode three α-helices with the second and third helix forming a helix–turn–helix conformation to bind to the target DNA. The conserved regularly spaced three tryptophan residues in the R2 repeat and the twotryptophan and the phenylalanine residue in the R3 repeat are indicated by arrows in figure 4.8.

Table 4.3: MYB cDNA isolated from different tissues of *Dendrobium hybrida*

Sequence name	Primer	[a]Tissue(s)	[b]Number of clones
Dh1leaf1	Dh1-Myb	leaf	1
Dh1leaf2	Dh1-Myb	leaf	1
Dh1leaf4	Dh1-Myb	leaf	1
Dh1leaf10	Dh1-Myb	leaf	1
Dh1leaf3	Dh1-Myb	leaf	4
Dh1leafAS1	Dh1-Myb	leaf	1
Dh1SB1	Dh1-Myb	smallest-sized floral bud	2
Dh1SB3	Dh1-Myb	smallest-sized floral bud	2
Dh1petal1	Dh1-Myb	petal	1
Dh2miB1	Dh2-Myb	medium floral bud lip	3
Dh2miB3	Dh2-Myb	medium floral bud	1
Dh2miB4	Dh2-Myb	medium floral bud	1
Dh2leaf1	Dh2-Myb	leaf mature floral bud	4
Dh2leaf4	Dh2-Myb	leaf mature floral bud	
Dh2leaf2	Dh2-Myb	leaf	2
Dh2leaf6	Dh2-Myb	leaf	1
Dh2leaf7	Dh2-Myb	leaf	1
Dh2leaf8	Dh2-Myb	leaf	1
Dh2leaf9	Dh2-Myb	leaf	1
Dh2leaf10	Dh2-Myb	leaf	1
Dh2SB1	Dh2-Myb	smallest-sized floral bud	1
Dh2SB2	Dh2-Myb	smallest-sized floral bud	4
Dh2SB6	Dh2-Myb	smallest-sized floral bud	1
Dh2SB7	Dh2-Myb	smallest-sized floral bud	1
Dh2SB9	Dh2-Myb	smallest-sized floral bud	1
Dh2SB8	Dh2-Myb	smallest-sized floral bud lip	2
Dh2SB11	Dh2-Myb	smallest-sized floral bud	1
Dh2MB1	Dh2-Myb	mature floral bud leaf petal	4
Dh2MB2	Dh2-Myb	mature floral bud	2
Dh2MB3	Dh2-Myb	mature floral bud	1

Table 4.3: continued: *MYB* cDNA isolated from different tissues of *Dendrobium hybrida*

Sequence name	Primer	[a]Tissue(s)	[b]Number of clones
Dh2MB4	Dh2-Myb	mature floral bud	1
Dh2MB7	Dh2-Myb	mature floral bud	1
Dh2MBAS1	Dh2-Myb	mature floral bud	2
Dh2MBAS3	Dh2-Myb	mature floral bud	1
Dh2MBAS4	Dh2-Myb	mature floral bud	1
Dh2MBAS5	Dh2-Myb	mature floral bud	1
Dh2petalAS2	Dh2-Myb	petal	1

[a]RNAs were extracted from smallest-sized floral bud (0-0.5 cm), medium-sized floral bud (1-1.5 cm), mature floral bud (3-3.5 cm), lip, petal and leaf tissues of *Dendrobium hybrida*. Extracted RNAs were subjected to RT-PCR, followed by cloning.
[b]Number of different cDNA sequences from all tissues.

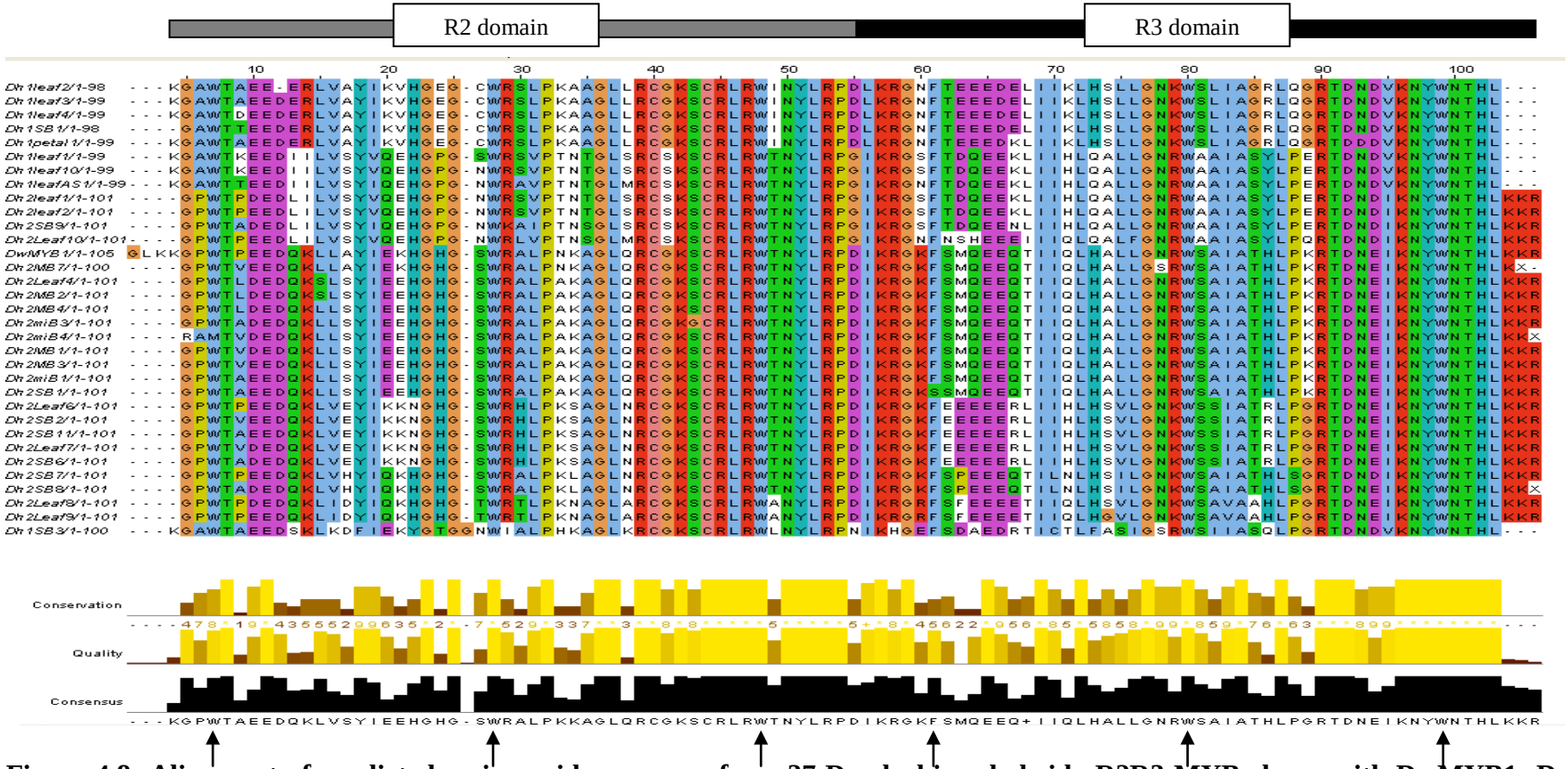
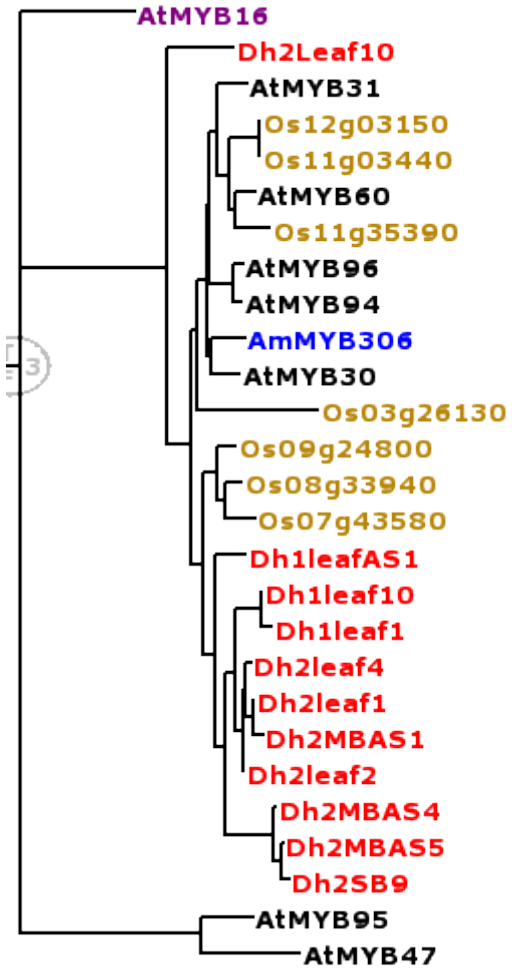


Figure 4.8: Alignment of predicted amino acid sequences from 37 Dendrobium hybrida R2R3-MYB clones with DwMYB1: Dendrobium sp. XMW-2002-1 MYB 1 (GenBank accession number: AAO49410). The bar above the alignment indicates the R2 and R3 domains, underlined in grey and black, respectively. The majority consensus sequences are highlighted and the conservation of each amino acid is shown below the alignment. The region of R2R3 domains of *Dendrobium hybrida* sequences are defined according to DwMYB1: *Dendrobium sp. XMW-2002-1* MYB 1 (GenBank accession number: AAO49410), whereas *Dendrobium hybrida* sequences indicate the tissue and primer pair used in RT-PCR amplification as shown in table 4.3. Arrows indicate the conserved regularly spaced three tryptophan residues in the R2 repeat and the two tryptophan and the phenylalanine residue in the R3 repeat.

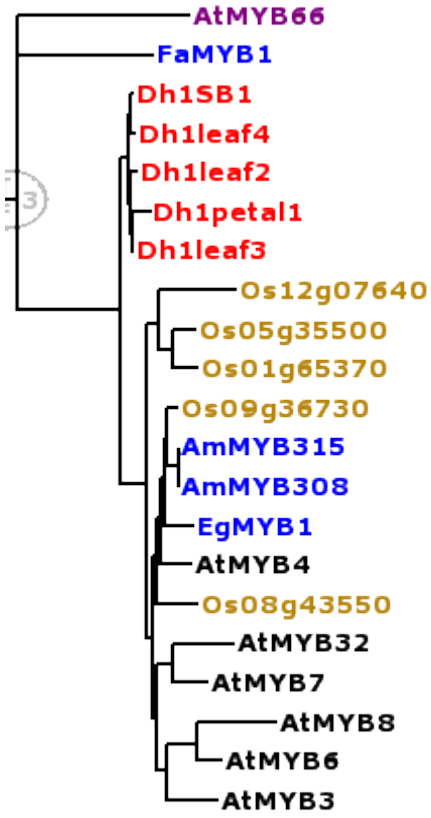
4.1.8 Phylogenetic analysis of R2R3 MYB sequences from *Dendrobium hybrida*

Phylogenetic analysis of the 37 putative R2R3-MYB protein sequences from *Dendrobium hybrida* with MYB proteins from *Arabidopsis thaliana*, *Oryza sativa*, *Antirrhinum majus*, *Gossypium hirsutum*, *Fragaria ananassa*, *Eucalyptus gunnii* and *Lentinula edodes* were carried out using Interspecies Transcription Factor Function Finder (IT3F) (http://jicbio.bbsrc.ac.uk/IT3F/). The R2R3 proteins were divided into 24 subgroups. This analysis revealed that MYB proteins from *Dendrobium hybrida* are clustered into six subgroups (subgroup 1, 4, 9, 10, 11 and 14) as shown in figure 4.9.

A.


AtMYB16
Dh2Leaf10
AtMYB31
Os12g03150
Os11g03440
AtMYB60
Os11g35390
AtMYB96
AtMYB94
AmMYB306
AtMYB30
Os03g26130
Os09g24800
Os08g33940
Os07g43580
Dh1leafAS1
Dh1leaf10
Dh1leaf1
Dh2leaf4
Dh2leaf1
Dh2MBAS1
Dh2leaf2
Dh2MBAS4
Dh2MBAS5
Dh2SB9
AtMYB95
AtMYB47

B.


AtMYB66
FaMYB1
Dh1SB1
Dh1leaf4
Dh1leaf2
Dh1petal1
Dh1leaf3
Os12g07640
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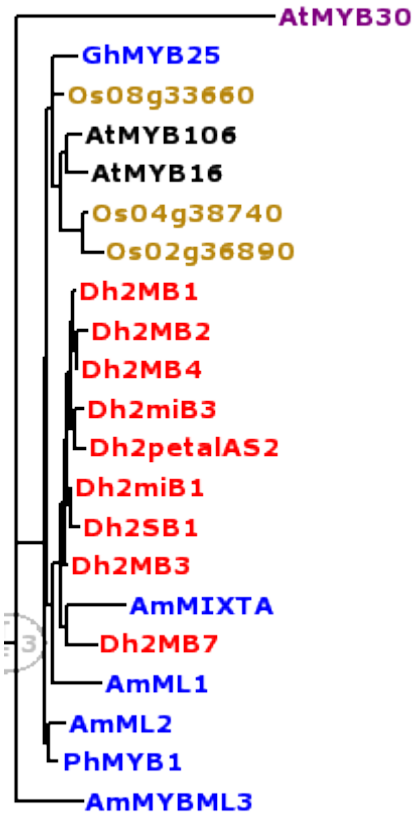
Os01g65370
Os09g36730
AmMYB315
AmMYB308
EgMYB1
AtMYB4
Os08g43550
AtMYB32
AtMYB7
AtMYB8
AtMYB6
AtMYB3

C.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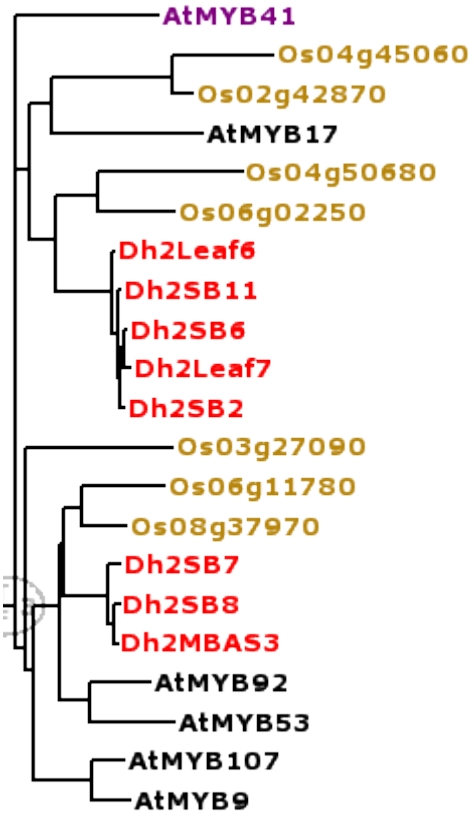
AtMYB106
AtMYB16
Os04g38740
Os02g36890
Dh2MB1
Dh2MB2
Dh2MB4
Dh2miB3
Dh2petalAS2
Dh2miB1
Dh2SB1
Dh2MB3
AmMIXTA
Dh2MB7
AmML1
AmML2
PhMYB1
AmMYBML3

D.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AtMYB17
Os04g50680
Os06g02250
Dh2Leaf6
Dh2SB11
Dh2SB6
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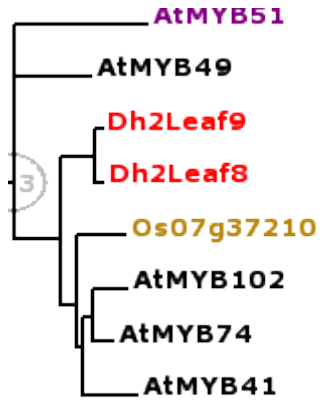
Dh2SB2
Os03g27090
Os06g11780
Os08g37970
Dh2SB7
Dh2SB8
Dh2MBAS3
AtMYB92
AtMYB53
AtMYB107
AtMYB9

E.



F.

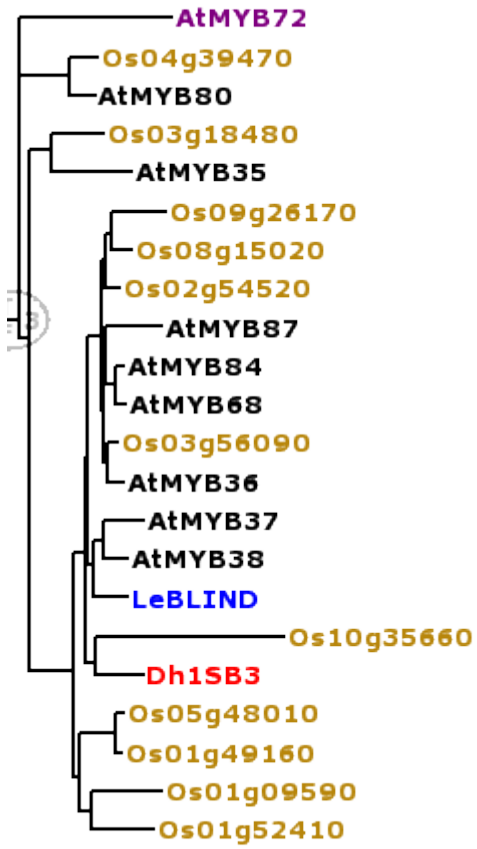


Figure 4.10: Phylogenetic relationship between Dendrobium hybrida R2R3-MYB proteins and R2R3-MYB proteins of Arabidopsis thaliana (At), Oryza sativa (Os), Antirrhinum majus (Am), Gossypium hirsutum (Gh), Fragaria ananassa (Fa), Eucalyptus gunnii (Eg) and Lentinula. Protein names of Arabidopsis, rice, and *Dendrobium hybrida* were drawn in black, yellow and red, respectively. (A). Phylogenetic tree of subgroup 1 contained AtMYB30 as a subgroup identifier and AtMYB16 as an out group. (B). Phylogenetic tree of subgroup 4 contained AtMYB32 as a subgroup identifier and AtMYB66 as an out group. (C). Phylogenetic tree of subgroup 9 contained AtMYB16 as a subgroup identifier and AtMYB30 as an out group. (D). Phylogenetic tree of subgroup 10 contained AtMYB107 as a subgroup identifier and AtMYB41 as an out group. (E). Phylogenetic tree of subgroup 11 contained AtMYB41 as a subgroup identifier and AtMYB51 as an out group. (F). Phylogenetic tree of subgroup 14 contained AtMYB36 as a subgroup identifier and AtMYB72 as an out group. A complete list of accession numbers for these sequences is provided in Appendix 1.

4.2 Cloning and phylogenetic analysis of full length *Dendrobium hybrida R2R3 MYB* cDNA

4.2.1 Cloning of full length *DhMYB* cDNA

DhMYB7 which was grouped together with AmMixta in subgroup 9 was selected and the partial cDNAsequence of *DhMYB7* was used as a template to design a new more specific PCR primers and for 5' RACE and 3' RACE to obtain a full length cDNA. A 1338 bp 3' RACE product was obtained using gene specific primer 1 and a primer complementary to the poly (A) tail whereas a 418 bp 5' RACE product was amplified using gene specific primer 2 and GeneRacer™ 5′ primer as shown in figure 4.11. Chromatograms of the sequence are shown in Appendix 1 and 2. A 1,487 bp sequence of *DhMYB* cDNA (accession number: JX846911) was obtained by joining the sequences from 5' RACE clones and 3' RACE clones using Bioedit software. Subsequently, Basic Local Alignment Search Tool (BLAST) analysis showed that the newly isolated *MYB* cDNA was highly identical (97%; 1408/1487 identity) to *DwMYB1* mRNA (accession number: AF485892.1), and 94% identical (1043/1487 identity) to *DcMYBML1* mRNA (accession number: GQ981643.1), as shown in table 4.4.

Table 4.4: BLAST result of full length MYB cDNA sequence with sequences in NCBI database

Accession	Description	[a]Query coverage	[b]Identity score	[c]E value	[d]Max ident
AF485892.1	*Dendrobium* sp. XMW-2002-1 MYB1 (MYB1) mRNA, complete cds	97%	1408/1456	0.0	97%
GQ981643.1	*Dendrobium crumenatum* MYBML1 mRNA, complete cds	73%	1043/1110	0.0	94%

[a]Query coverage indicated the percentage of *Dendrobium hybrida* cDNA was aligned.
[b]Identity score indicates the total nucleotides of *Dendrobium* sp. XMW-2002-1 MYB1 (MYB1) mRNA and *Dendrobium crumenatum* MYBML1 mRNA that are identical with those of the *Dendrobium hybrida* cDNA.
[c]E value zero indicated significant alignment between *Dendrobium hybrida* full length *MYB* cDNA with *Dendrobium* sp. XMW-2002-1 MYB1 (MYB1) mRNA and *Dendrobium crumenatum* MYBML1 mRNA.
[d]Max ident showed the percentage of *Dendrobium hybrida* full length *MYB* cDNA match up with *Dendrobium* sp. XMW-2002-1 MYB1 (MYB1) mRNA and *Dendrobium crumenatum* MYBML1 mRNA within the query coverage.

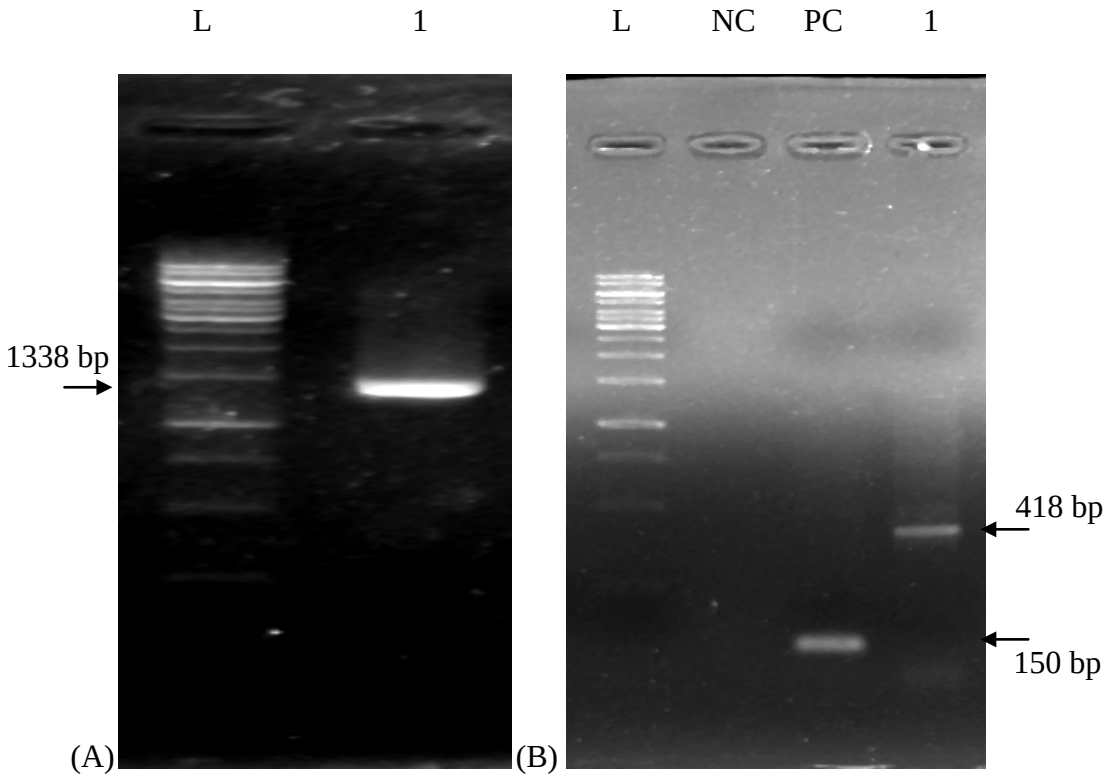


Figure 4.11 Agarose gel analysis of 5' and 3' RACE PCR products. (A) Amplification of partial 3' region of *MYB* cDNA using mature floral bud (3-3.5 cm) cDNA as template. Lane 1: 3' RACE PCR product. (B) Amplification of partial 5' region of *MYB* cDNA using mature floral bud (3-3.5 cm) cDNA as template. Lane NC: negative control of PCR; lane PC: positive control; lane 1: 5' RACE PCR product. For all gels, lane L: Fermentas 1kb DNA marker.

4.2.2 Phylogenetic analysis of full length *Dendrobium hybrida* R2R3-MYB

Full length sequence of DhMYB7 (named DhMYB) from *Dendrobium hybrida* and known R2R3-MYB sequences from diverse plants within subgroup 9 were used for further phylogenetic analysis. DhMYB grouped together with DwMYB1 and DcMYBML1 whilst its sister group contained AmMIXTA and AmMYBML1 as shown in figure 4.12. In addition, alignment of 493 deduced amino acid sequences isolated from *Dendrobium hybrida* with MYB sequences from subgroup 9 revealed high sequence similarity within the R2R3 domain at the N-terminus (amino acid 43 to 167) as shown in figure 4.13

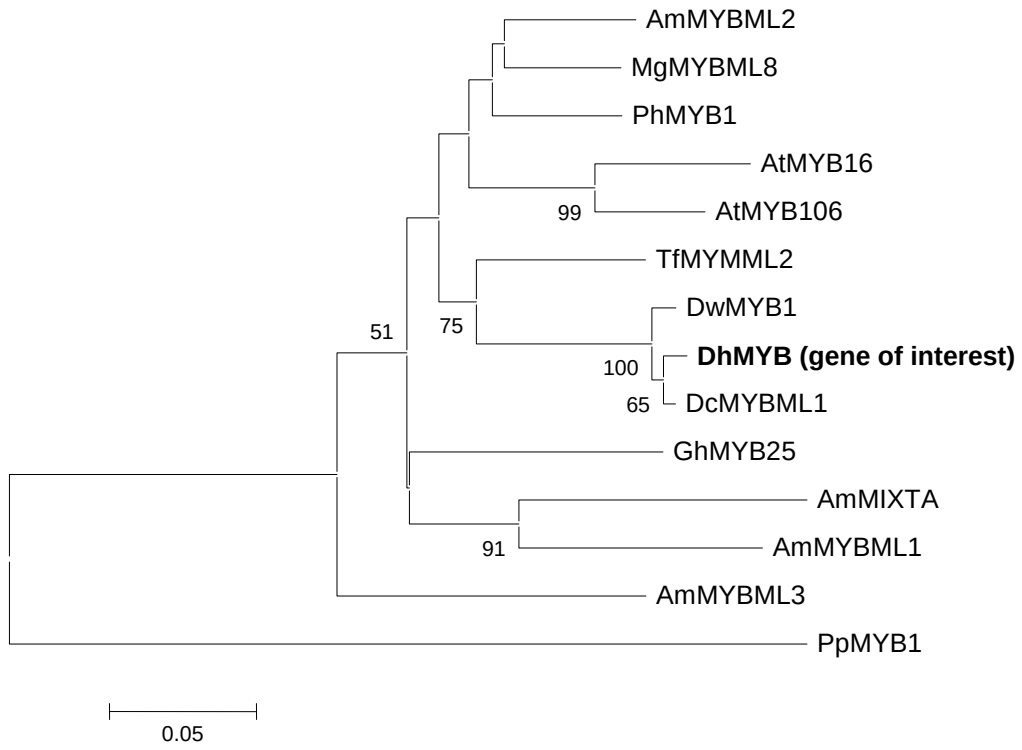
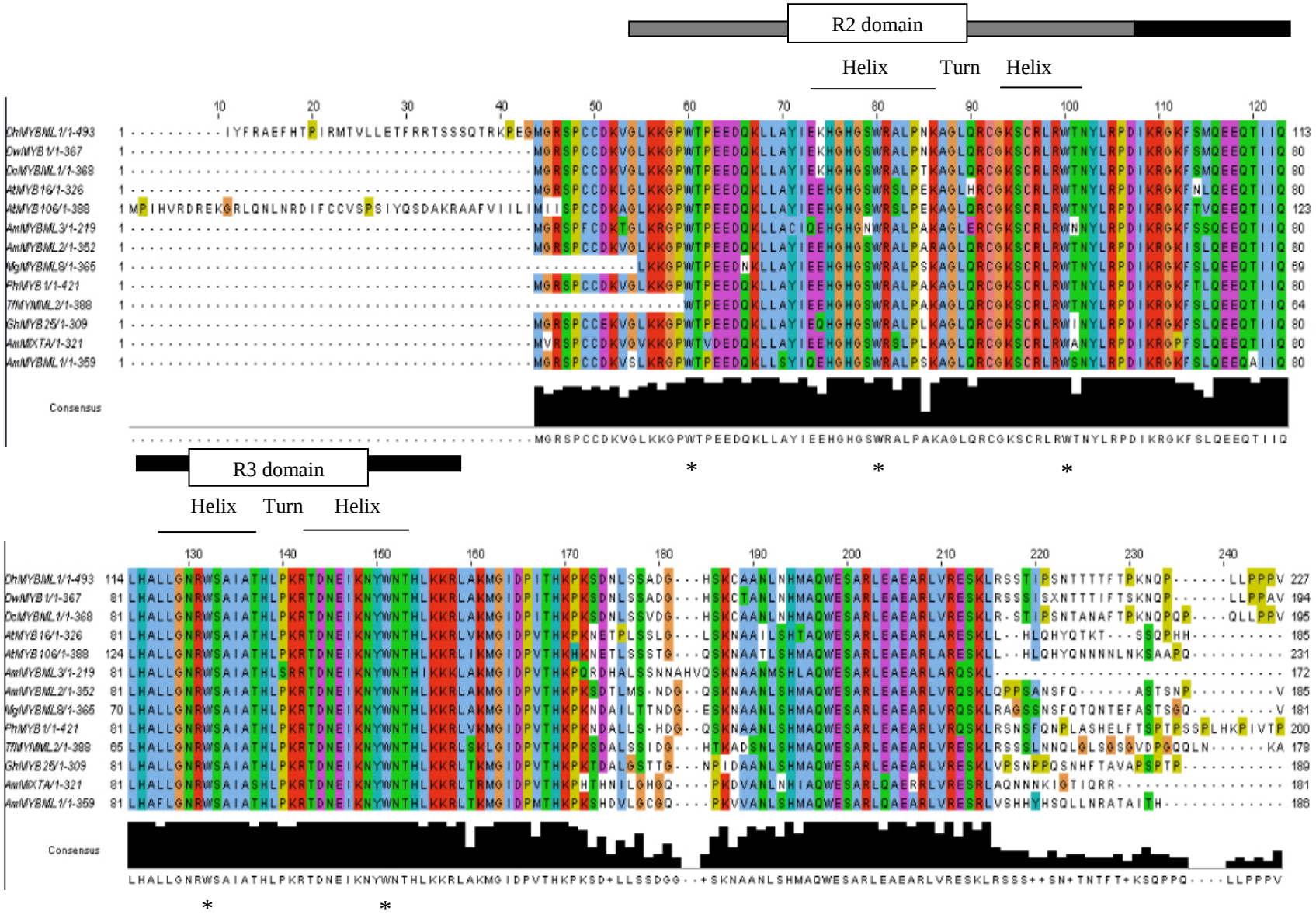


Figure 4.12: Phylogenetic relationship of DhMYB and R2R3-MYB proteins belonging to subgroup 9. AmMIXTA (accession number: CAA55725.1), MIXTA-LIKE MYB gene AmMYBML1 (accession number: CAB43399.1), AmMYBML2 (accession number: AAV70655) and AmMYBML3 (accession number: AAU13905) from *Antirrhinum majus*; AtMYB16 (accession number: AED92146.1) and AtMYB106 (accession number: AEE73615.1) from *Arabidopsis thaliana*; PhMYB1 (accession number: CAA78386.1) from *Petunia hybrida*; DcMYBML1 (accession number: ADD64500.1) from *Dendrobium crumenatum*, DwMYB1 (accession number: AAO49410.1) from *Dendrobium* sp.; TfMYMML2 (accession number: ACT78694) from *Thalictrum filamentosum*; MgMYBML8 (accession number: ADV29952.1) from *Mimulus guttatus pop-variant IM62*; DhMYB from *Dendrobium hybrida.* Bootstrap values are indicated at the nodes of the branches (values inferior to 50% have been omitted). PpMYB1 (accession number: ACA33839) was used as an outgroup to root the tree. The scale bar represents 0.05 substitutions per site.


R2 domain
Helix
Turn
Helix
R3 domain
Helix
Turn
Helix
Consensus
Consensus

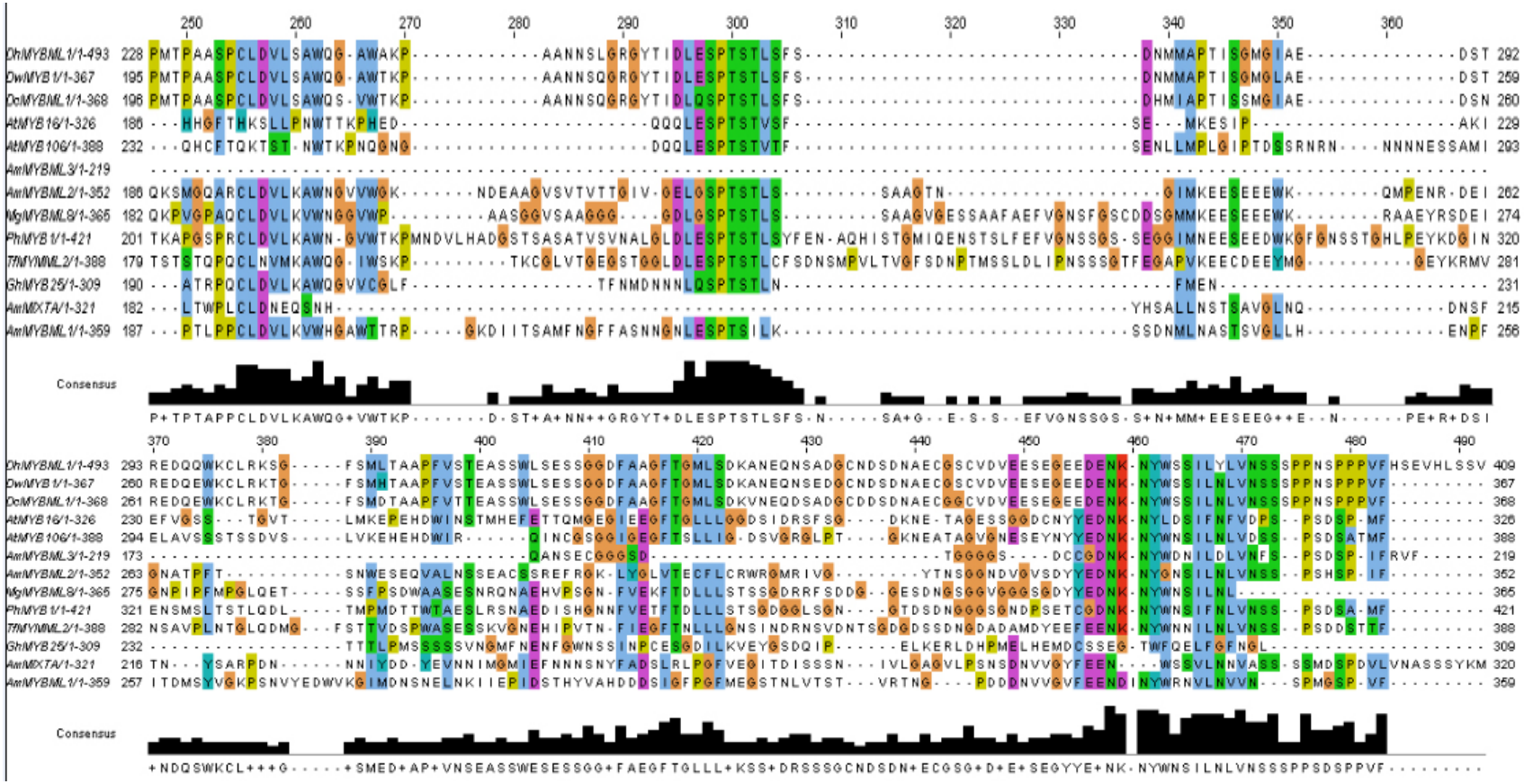


Figure 4.13: Amino acid sequence alignment of DhMYB with other MYBs in subgroup 9 by using clustalW. The R2 and R3 domains are underlined in grey and black, respectively whereas the positions of helix-turn-helix region are indicated by lines above the alignment. The majority consensus sequences are highlighted and the conservation of each amino acid is shown in the bar below the alignment. Dashes indicate gaps; asterisks indicate regularly spaced tryptophan residues. The R2R3 domains, helix-turn-helix regions and regularly spaced tryptophan residues are defined according to R1R2R3 type c-MYB protein (GenBank accession number AAA48962).

4.3 Growth and development of *Dendrobium hybrida* floral buds

For convenience for comparison during gene expression experiments, floral buds and flowers from *Dendrobium bobby messina* x *Dendrobium chao phraya* were divided into 9 developmental stages from emerged bud until fully opened flower, based on physical characteristics (size and appearance) in, as shown in table 4.5. *Dendrobium hybrida* emerged buds typically took 6 days to reach 0.5 cm in length; 11 days to reach 1 cm in length; 15 days to reach 1.5 cm in length, 19 days to reach 2 cm in length; 22 days to reach 2.5 cm in length; 25 days to reach 3 cm in length; 29 days to reach 3.5 cm in length; 30 days to reach unfolding of petals and sepals and 32 days to reach anthesis. The flower development followed a concave down increasing curve with R^2 value 0.967 as shown in figure 4.14.

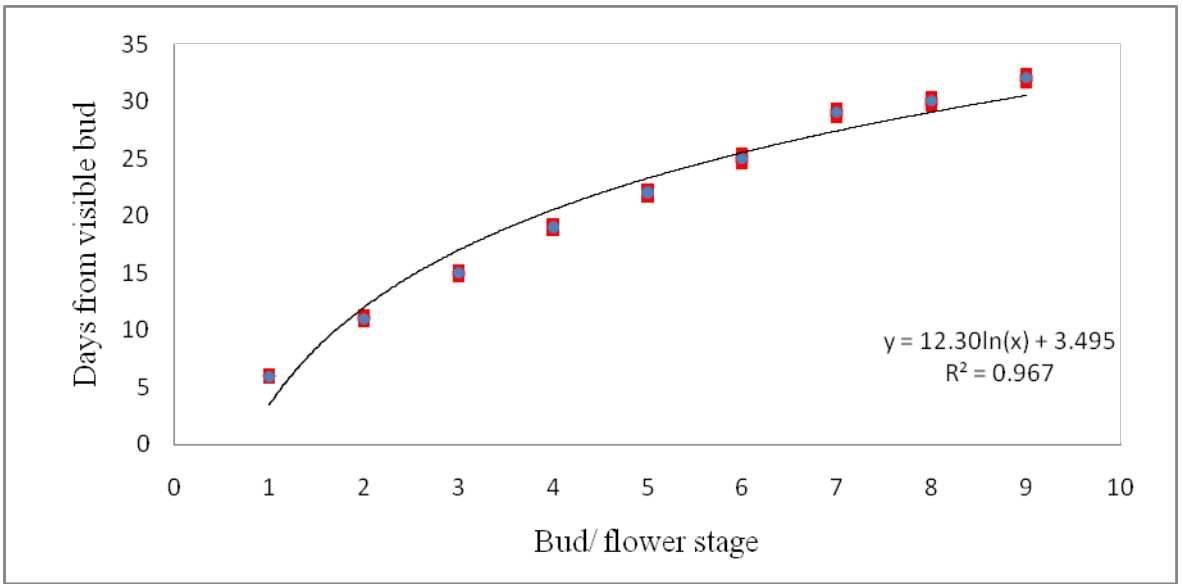
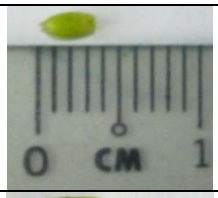
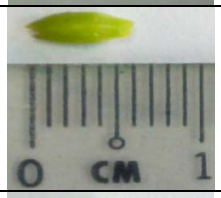


Figure 4.14: The relationship between bud or flower stage and mean days taken to reach each stage from emerged bud. Squares indicate mean values of observations from 30 buds. Vertical range bars indicate standard error of the mean. Model simulation is indicated by the line.

Table 4.5: Physical characteristics of the bud and flower stages of *Dendrobium bobby messina* x *Dendrobium chao phraya*

Bud/flower stage	Bud length (cm)	Image	Description
1	0-0.5		Immature bud, light green adaxial and abaxial surface
2	0.5-1.0		Small bud, light green adaxial and abaxial surface
3	1.0-1.5		Medium-small, adaxial surface still green, most of the abaxial surface turned purple
4	1.5-2.0		Medium size, adaxial surface of perianth turned purple
5	2.0-2.5		Medium-large, dark purple on abaxial surface, purple on adaxial surface
6	2.5-3.0		Large, unopened, dark purple on both adaxial and abaxial surfaces of perianth
7	3.0-3.5		Mature bud, unopened, dark purple on both adaxial and abaxial surfaces of perianth
8	Unfolding flower		Flowers just opened with lateral sepals unfolded
9	Fully open flower		Flowers fully open, dark purple perianth

4.4 Quantification of the expression levels of the *DhMYB* cDNA in different floral bud stages

To investigate the suitable floral bud stage for functional study of *DhMYB* cDNA, transcript levels in floral buds from stage 1 to stage 7 were measured using RT-qPCR. Prior to using the $\Delta\Delta C_T$ method for quantification in RT-qPCR, a validation experiment was performed. Efficiencies of *DhMYB* cDNA target gene and *β-actin* cDNA reference gene were approximately equal as the absolute value of the slope of log input amount versus ΔC_T was 0.082 (i.e. lower than the required 0.1 maximum) as shown in figure 4.15.

DhMYB cDNA was observed for all floral bud stages, with highest expression levels found in stage 6 whereas the lowest transcript level was found in stage 1. The transcript level of *DhMYB* increased gradually from stage 1 to stage 6 and decreased at stage 7 as shown in figure 4.16. One way ANOVA analysis and multi-comparison analysis (Tukey HSD test) were carried out from the average results of three independent experiments. According to a Tukey HSD comparison test, stage 1 to stage 7 show differences in *DhMYB* expression level ($P < 0.05$) as shown in appendix 3.

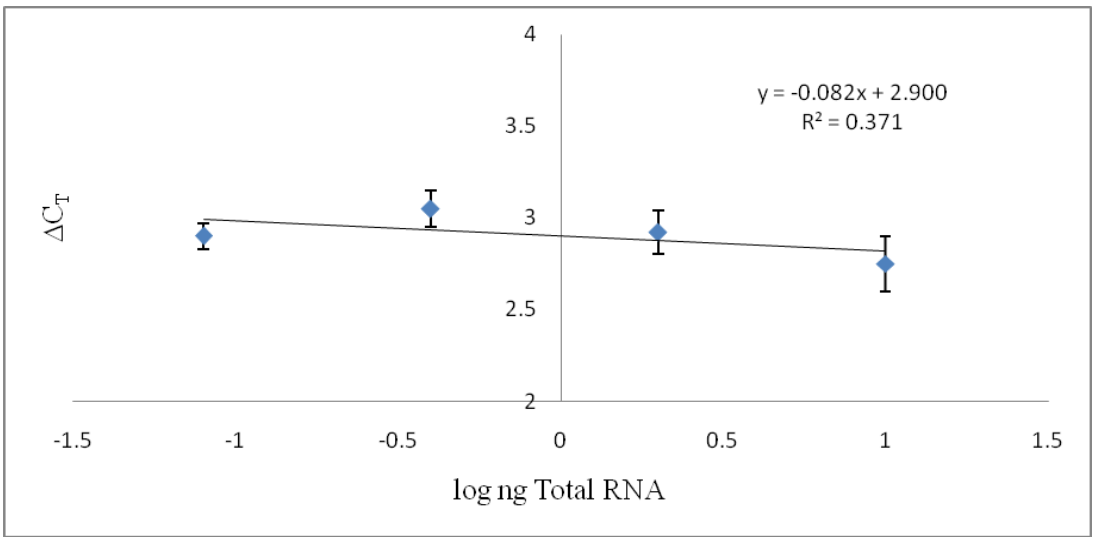


Figure 4.15: Plot of log total RNA versusΔCT . Square indicate mean of ΔC_T value from 3 replicates, vertical lines indicate standard error of the mean.

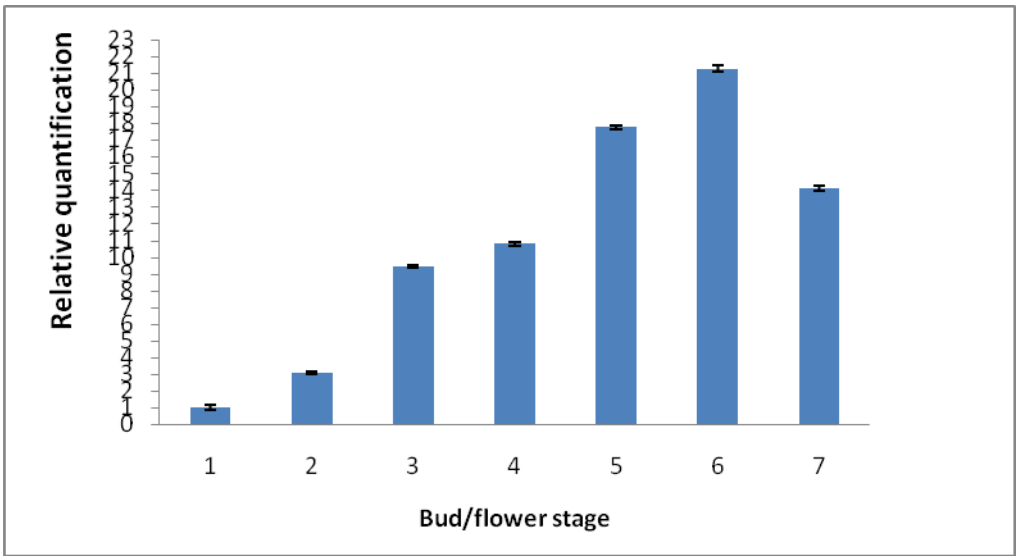


Figure 4.16: Quantification of expression levels of the DhMYB cDNA in floral buds from stage 1 to stage 7. Relative expression of *DhMYB* cDNA was obtained by dividing the average number of *DhMYB* transcript copies by the copy number of *Dendrobium hybrida β-actin* (endogenous control) for the same tissue. The lowest transcript level (*DhMYB* cDNA in stage 1) was then set to a value of 1 and subsequently expression levels are reported relative to this number. Bars indicate standard error from amplification of 3 replicates of *DhMYB* cDNA samples.

4.5 Cell shapes of the adaxial epidermis of the perianth of *Dendrobium hybrida*

Adaxial epidermal cell shapes of lip, petal and sepal of *Dendrobium hybrida* at stage 9 were observed under scanning electron microscopy. The lip in *Dendrobium hybrida* was more complex compared to the sepal and petal in terms of epidermal cell shapes. There are two domains in sepal and petal according to two different types of epidermal cell shapes which were flattened epidermal cells at domain 1 and flattened epidermal cells with rectangular bases at domain 2 as shown in table 4.6. In contrast, there were seven domains with seven different types of epidermal cell shapes on the adaxial surface of the lip, which were conical - papillate epidermal cells at domain 1; flattened epidermal cells at domain 2; epidermal cells with a single, central outgrowth of the outer, cuticularised wall at domain 3; random cellular outgrowths at domain 4; regular striations at domain 5; flattened epidermal cells with rectangular bases and random conical cells on the surface at domain 6; flattened epidermal cells with rectangular bases at domain 7 as shown in table 4.6.

Table 4.6: Various shapes of adaxial epidermal cells in the perianth of *Dendrobium hybrida*

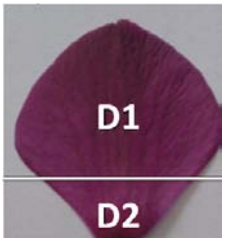
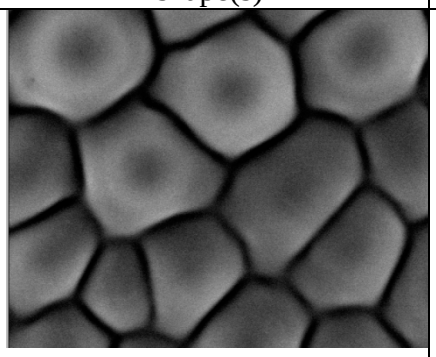
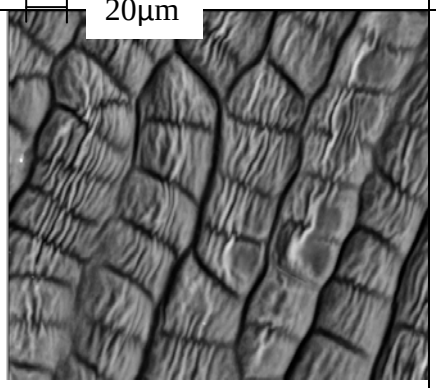
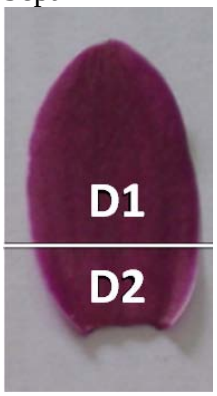
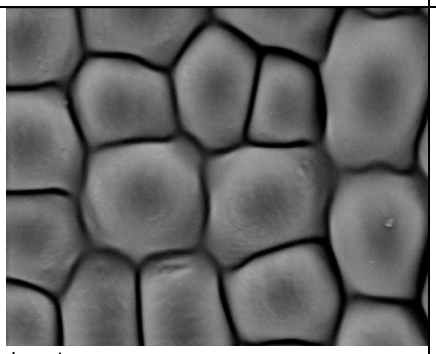
Floral organ	Domain	[a] Adaxial epidermis cell shape(s)	Description
Petal 	Domain 1 (D1)		Flattened epidermal cells. Variation in base diameter of epidermal cells.
	Domain 2 (D2)	 20µm	Flattened epidermal cells with rectangular bases. Variation in base diameter of epidermal cells.
Sepal 	Domain 1 (D1)	 20µm	Flattened epidermal cells. Variation in base diameter of epidermal cells.

Table 4.6 continued: Various shapes of adaxial epidermal cells in the perianth of *Dendrobium hybrida*

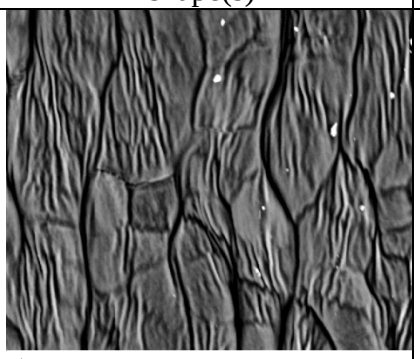
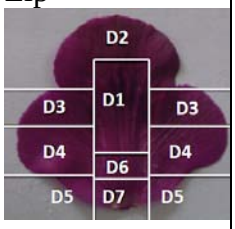
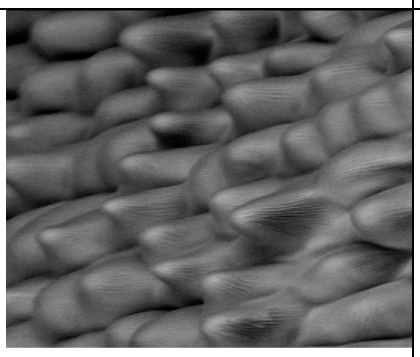
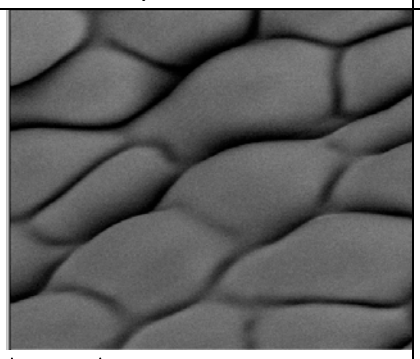
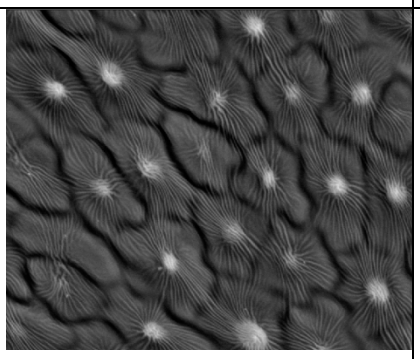
Floral organ	Domain	Adaxial epidermis cell shape(s)	Description
Sepal	Domain 2 (D2)	 20µm	Flattened epidermal cells with irregular bases. Variation in base diameter of epidermal cells.
Lip 	Domain 1 (D1)	 20µm	Conical – papillate epidermal cells. Variations in the steepness of the cone of conical cells.
	Domain 2 (D2)	 20µm	Flattened epidermal cells. Variation in base diameter of epidermal cells.
	Domain 3	 20µm	Epidermal cells shows a single, central outgrowth of the outer, cuticularised wall. Variation in base diameter of epidermal cells.

Table 4.6 continued: Various shapes of adaxial epidermal cells in the perianth of *Dendrobium hybrida*

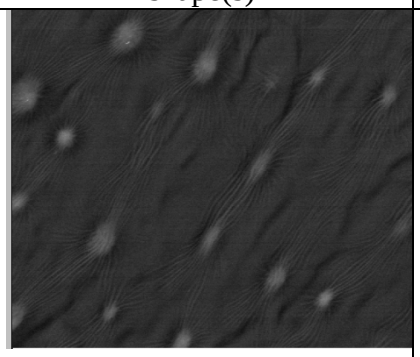
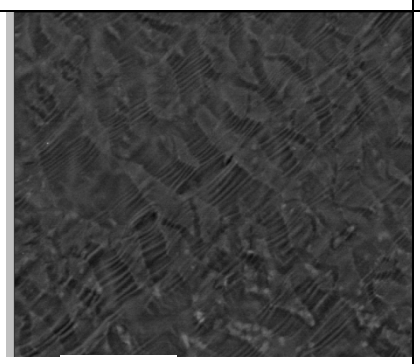
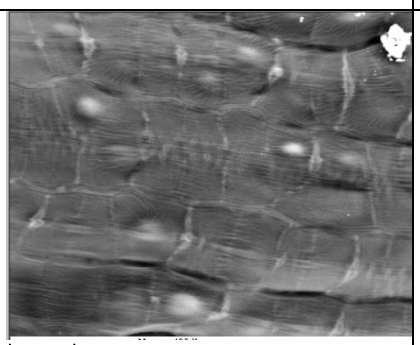
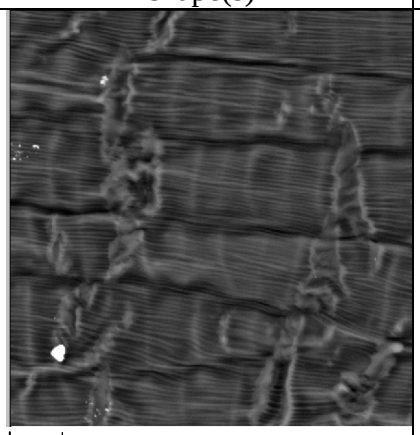
Floral organ	Domain	Adaxial epidermis cell shape(s)	Description
Lip	Domain 4	 20µm	Random cellular outgrowths on the adaxial lip surface. Variation in base diameter of epidermal cells.
	Domain 5	 20µm	Regular striations within the epidermal cells. Variation in base diameter of epidermal cells.
	Domian 6	 20µm	Flattened epidermal cells with rectangular bases. Random conical cells on the surface. Variation in base diameter of epidermal cells.

Table 4.6 continued: Various shapes of adaxial epidermal cells in the perianth of *Dendrobium hybrida*

Floral organ	Domain	Adaxial epidermis cell shape(s)	Description
Lip	Domain 7	 20μm	Flattened epidermal cells with rectangular bases. Variation in base diameter of epidermal cells.

[a]Adaxial epidermis cell shapes were captured on fresh samples using variable pressure scanning electron microscopy with 400 x magnification.

4.6 Functional study of *DhMYB* cDNA in *Dendrobium hybrida*

4.6.1 Isolation and characterisation of partial *DhMYB* cDNA

Specific forward and reverse primers of *DhMYB* cDNA were flanked with *Not* I and *Nco* I restriction enzyme sequence, respectively to amplify a partial sequence of *DhMYB* cDNA at the 3' region (from nucleotide 770 to nucleotide 1198). This primer pair was tested with annealing temperatures of 48 ^{0}C to 64 ^{0}C as shown in figure 4.17 (A). Annealing temperature of 48 ^{0}C, 49.4 ^{0}C, 51.2 ^{0}C and 53.5 ^{0}C gave a PCR product of 450 bp in size whereas an annealing temperature of 48 ^{0}C produced the sharpest bands thus was selected for further use. A single PCR product from mature floral bud (3.0-3.5 cm) tissue, as shown in figure 4.17 (B), was ligated into L4440 vector followed by transformation into *E. coli* HT115 strain. Seven colonies were selected for colony PCR and three insert containing plasmids as shown in figure 4.17 (D) were sequenced. PCR products (450 bp) from colony selection for the transformation are shown in figure 4.17 (C). The quality of plasmid DNA was assessed from the ratio of the absorbance at 260 nm/ absorbance at 280 nm and is shown in table 4.7.

Table 4.7: Quality of isolated plasmid harbouring *Dendrobium hybrida* cDNA.

Plasmid	Concentration (ng/ μl)	Ratio of absorbance: 260/280
1	464.2	1.87
2	402.1	1.88
3	234.3	1.87

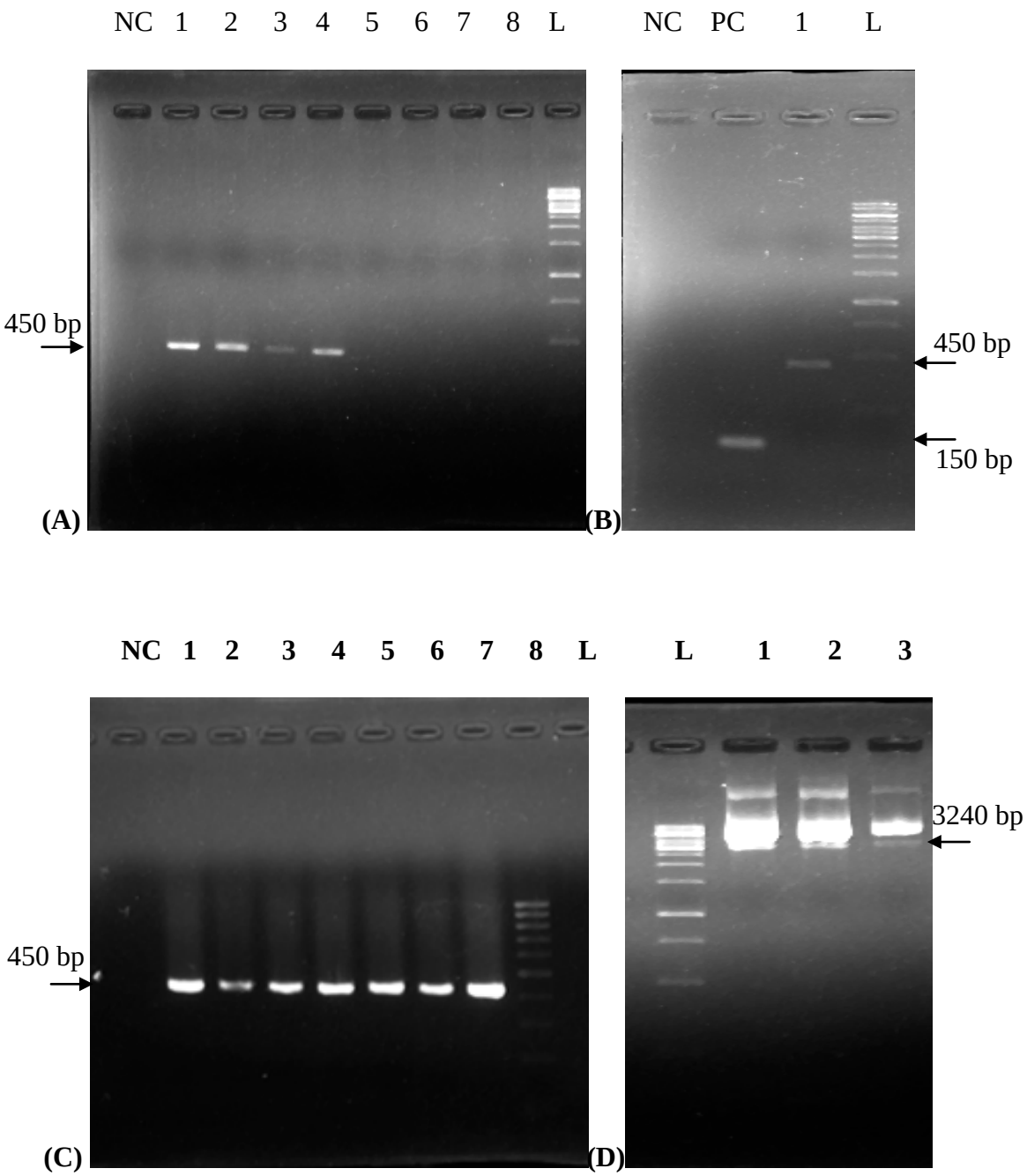


Figure 4.17: Isolation and characterisation of partial DhMYB cDNA. (A) Agarose gel electrophoresis analysis of gradient PCR using DhMYB primer. Lane 1: annealing temperature 48 ^{0}C: lane 2; 49.4 ^{0}C; lane 3: 51.2 ^{0}C; lane 4: 53.5 ^{0}C; lane 5: 56.1 ^{0}C; lane 6: 58.8 ^{0}C; lane 7: 61.5 ^{0}C; lane 8: 64 ^{0}C. (B) Agarose gel electrophoresis analysis of purified *DhMYB* PCR product. Lane PC: positive control of the amplification by using β-actin primer pair; lane 1: purified *DhMYB* PCR product. (C) Colony PCR was carried out to determine the colonies that harbour the sequence of interest. PCR products from colonies 1 to 7 were loaded in lanes 1 to 7 respectively. (D) Plasmid isolated from the clones. Plasmid DNA from clones 1 to 3 was loaded in lanes 1 to 3 respectively. For all gels, NC: negative control of amplification; lane L: Fermentas 1kb DNA marker.

4.6.2 Production of *DhMYB*-derived dsRNA using an RNase-deficient *E. coli* strain

E. coli strain HT115 (DE3) was transformed with a single plasmid, pL4440/partial *DhMYB* cDNA, designed to express a dsRNA containing partial *DhMYB* sequences under the control of two copies of the T7 promoter, placed on opposite strands at each end of the insert. Upon induction with 0.4 mM IPTG, the optimum OD_{600} from range 0.5 to 1.0 was determined by measuring the concentration of induced dsRNA, as shown in table 4.8 and figure 4.18A. Based on this, the optimal OD_{600} of 0.7 was selected for large scale dsRNA preparation. Next, bacterial crude extracts were treated with RNase A and DNase I to remove single-stranded RNA and genomic DNA respectively as shown in figure 4.18B. Purified dsRNA was subjected to RT-PCR and the products sequenced. The amplified sequence of 180 bp was 100 % identical to partial *DhMyb* cDNA (from nucleotide 792 to nucleotide 972 of gene accession number: JX846911). RT-PCR product from extracted dsRNA is shown in figure 4.18 C.

Table 4.8: Quality of isolated bacterial crude extracts harbouring DhMYB dsRNA.

OD_{600}	Concentration (µg/ µl)	Ratio of absorbance: 260/280
0.5	1.4244	1.89
0.6	1.4371	1.89
0.7	1.6569	1.8
0.8	1.379	1.91
0.9	1.2621	1.79
1.0	1.2487	1.81

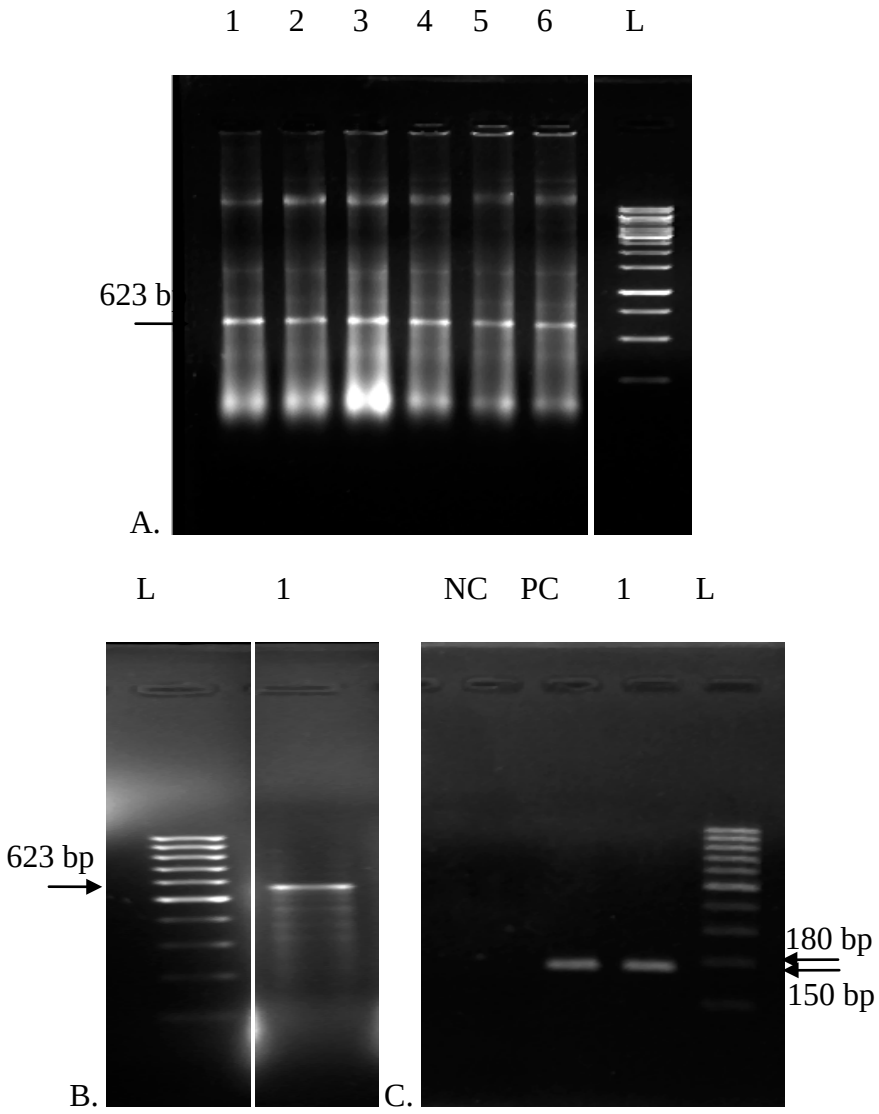


Figure 4.18: Optimization and analysis of the bacterially expressed DhMYB dsRNA in an E. coli strain deficient for RNase III. A. Optimum OD_{600} was tested from the range 0.5 to 1.0 to obtain the highest concentration of dsRNA. Lane 1: OD_{600} = 0.5; lane 2: 0.6; lane 3: 0.7; lane 4: 0.8; lane 5: 0.9; lane 6: 1.0 and L: Fermentas 1kb DNA marker. B. Bacterial crude extracts after treatment with RNase A and DNase I. Lane 1: purified bacterially expressed *DhMYB* dsRNA; lane L: Fermentas 100 bp DNA marker. C. RT-PCR product from extracted dsRNA. Lane 1: purified RT-PCR product; lane NC: negative control of amplification; lane PC: positive control of amplification; lane L: Fermentas 100 bp DNA marker.

4.6.3 RNAi knock down of *DhMYB* expression

4.6.3.1 Analysis of expression of *DhMYB* and phenotype of *Dendrobium hybrida* floral buds treated with *DhMYB* dsRNA

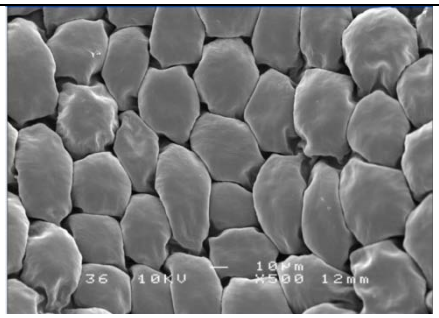
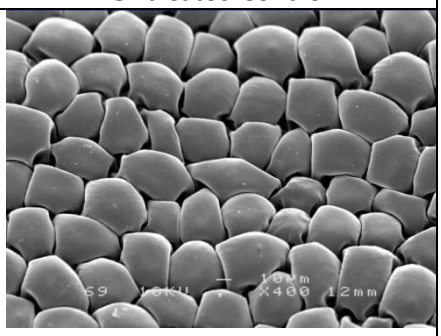
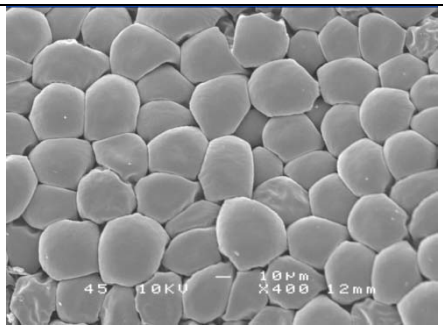
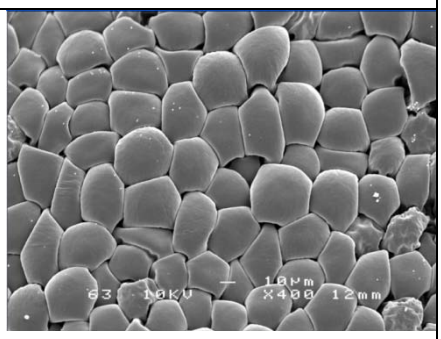
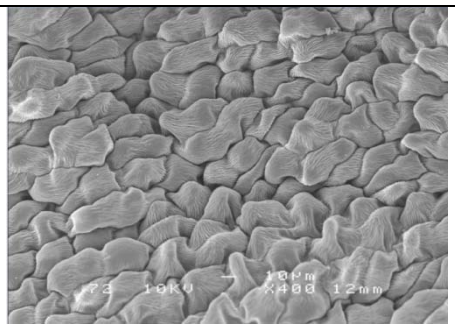
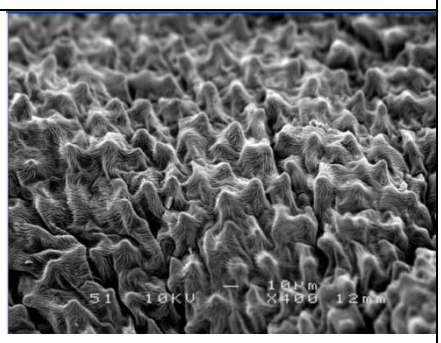
Dendrobium hybrida buds were treated at stage 1 and repeated treatment was done in 5 interval days. Untreated control buds and buds treated with *DhMYB* dsRNA were harvested at stages 5, 6 and 7, which were at 22 days post-treatment (dpt), 25 dpt and 29 dpt, respectively. Total RNAs were extracted and expression levels of *DhMYB* were analysed by RT-qPCR. Two way ANOVA analysis and multi-comparison analysis (Tukey HSD test) were carried out using the average results of three independent experiments. According to a Tukey HSD comparison test, transcript levels of treated buds were significantly different to transcript levels of untreated buds ($P < 0.05$) as shown in table 4.9 and appendix 7. *Dendrobium hybrida* treated buds at stage 5, 6 and 7 were 3.3-fold, 4-fold and 2.4-fold down regulated, respectively when compared with untreated control. Images of floral buds were captured prior to RNA extraction. There is no obvious colour changes between buds treated with dsRNA and untreated buds as shown in figure 4.9 (A) and appendix 8.

4.6.3.2 Adaxial epidermal cells shape in the perianth of buds treated with *DhMYB* dsRNA

Dendrobium hybrida floral buds were treated with *DhMYB* dsRNA and allowed to grow until the flower fully opened. Adaxial epidermal cell shapes of domain 1 of sepal, petal and lip were observed under scanning electron microscopy and compared with those from untreated control flowers. There were no observable differences in adaxial epidermal cell shapes within domain 1 of sepal and petal, between untreated control and plants treated with *DhMYB* dsRNA, with both showing flattened epidermal cell shape. In contrast, the cell shape of adaxial epidermal cells within domain 1 of lip treated with *DhMYB* dsRNA differed from those of the matching control. The treated

flower had relatively flattened epidermal cells whilst those of the control were conical – papillate,as shown in table 4.19.

Table 4.9: Comparison between cell shape of adaxial epidermal cells from dsRNA treated and untreated perianth of *Dendrobium hybrida*.

Floral organ	[a]Treated with *DhMYB* dsRNA	[b]Untreated control
Sepal (Domain 1)		
Petal (Domain 1)		
Lip (Domain 1)		

[a]SEM image of adaxial epidermis cells shape of sepal, petal and lip treated with *DhMYB* dsRNA at 400 x magnification.
[b]SEM image of adaxial epidermis cells shape of sepal, petal and lip of untreated control at 400 x magnification.

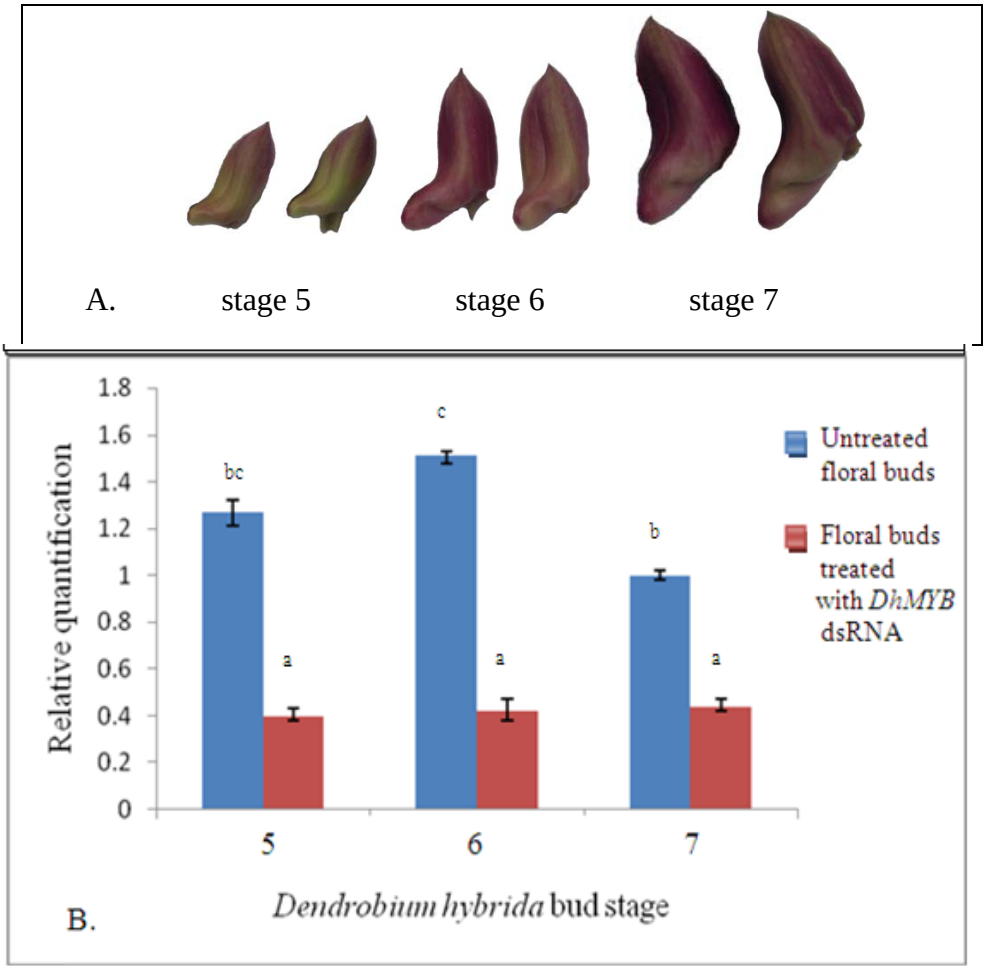


Figure 4.19: Gene expression and appearance of buds at after treatement with dsRNA. (a) Pairs of untreated buds (left for each pair) and buds treated with *DhMYB* dsRNA (right) at stage 5 (22dpt), 6(25dpt) and 7 (29 dpt) from left to right.. (b) Quantification of expression levels of the *DhMYB* and phenotypes of untreated buds and buds treated with *DhMYB* dsRNA at stage 5, 6 and 7. Relative expression of *DhMYB* was obtained by dividing the average number of *DhMYB* transcript copies by the copy number of *Dendrobium hybrida β-actin* (endogenous control) for the same tissue. The transcript level of *DhMYB* RNA in stage 7 untreated floral buds was then set to a value of 1 and subsequently expression levels are relative to this number. Bars indicate standard error from triplicate amplification of the *DhMYB* cDNA samples. [a] indicate transcript levels of *DhMYB* gene in treated buds at stage 5, 6 and 7 are significantly different from untreated control buds, at stage 5, 6 and 7 , P, <.05.b and c indicate transcript levels of *DhMYB* gene in untreated control buds at stage 6 and 7 are significantly different, P<,.05.

Chapter 5.0 Discussion

Current study was carried out to identify gene that play a role in cell shape development and flower colour in *Dendrobium hybrida*. The alternative approach, mechanical inoculation of crude extracts of bacterialled expressed *DhMYB* dsRNA was used.

5.1 Characterization of the partial *Dendrobium hybrida* R2R3MYB

R2R3MYBs are widely distributed in higher plants such as Arabidopsis, cucumber, maize, rice and populus and comprise one of the major families of regulatory proteins in plants (Li, *et al.*, 2012; Chen, *et al.*, 2006; Kranz, *et al.*, 1998; Du, *et al.*, 2012; Wilkins, *et al.*, 2009). A previous study reported four full length R2R3 MYB cDNAs and seventeen partial R2R3 MYB cDNAs in *Dendrobium* orchid hybrid Woo Leng, however these had unknown functions (Wu, *et al.*, 2003). In the current study, we were primarily interested in regulatory *R2R3 MYB* cDNAs that function in anthocyanin biosynthesis and determination of cell shape.

Sequence comparison of translated *Dendrobium hybrida* proteins with DwMYB1: *Dendrobium sp. XMW-2002-1* MYB 1 has revealed that they are R2R3 MYB family members due to the presence and characteristics of two repeat regions (Wu, *et al.*, 2003). These two repeat regions are constituted by two imperfect repeats of 50 amino acids. The R2 repeat has three regularly spaced tryptophan residues and the R3 repeat includes two tryptophan and one phenylalanine residue which encode three α-helices with the second and third helix forming a helix–turn–helix conformation to bind to the target DNA (Du, *et al.*, 2012; Wu, *et al.*, 2003; Stracke, *et al.*, 2001). We successfully identified and characterized 37 partial *Dendrobium hybrida R2R3MYB* cDNA through RT-PCR approach: 12 from leaves; 8 from small floral buds; 2 from medium-sized buds; 11 from mature floral buds; 2 from petals and 2 from lips. Notably, some of the MYB genes are expressed in various tissues and most are tissue specific.

For instance, DhMYB1 cDNA was detected in leaf, petal and mature floral bud tissues. However, the results in table 4.3 may not provide a comprehensive list as the genes that were identified from the tissues sampled in this study may not be representative of the whole genome of *Dendrobium hybrida*.

5.2 Phylogenetic analysis of *Dendrobium hybrida* R2R3MYB

Phylogenetic analysis of the R2R3MYB proteins has been studied in Arabidopsis (Dubos, *et al.*, 2010; Stracke, *et al.*, 2001), maize (Du, *et al.*, 2012; Hernandez, *et al.*, 2004), rice (Chen, *et al.*, 2006), grape (Matus, *et al.*, 2008) and soybean (Du, *et al.*, 2012). Moreover, the evolutionary relationship of R2R3 MYB genes within and among the different species has been extensively studied (Li, *et al.*, 2012 ; Chen, *et al.*, 2006). To get the clear image of the partial *Dendrobium hybrida* MYBs and their relationships with those from Arabidopsis, rice and Antirrhinum, translated *Dendrobium hybrida* R2R3MYB sequences were submitted to Interspecies Transcription Factor Function Finder (IT3F) (http://jicbio.bbsrc.ac.uk/IT3F/) (Bailey, *et al.*, 2008). With the data views provided in IT3F as shown in figure 4.9, it is possible to observe that the *Dendrobium hybrida* sequences are closely related to those from MYB genes across Arabidopsis, rice and Antirrhinum (Bailey, *et al.*, 2008).

R2R3 MYB proteins have been divided into 24 distinct subgroups according to the degree of similarity between their conserved DNA-binding domain as well as conserved amino acid motifs in their C-terminal domains (Li, *et al.*, 2012; Kranz *et al.*, 1998; Stracke *et al.*,2001). The conserved motifs of the established subgroups of MYB transcription factors may facilitate the identification of functional domains in *Dendrobium hybrida* MYB genes (Kranz *et al.*, 1998; Stracke *et al.*,2001; Perez-Rodriguez, *et al.*, 2005). Thirty-seven partial R2R3 MYBs were divided into 6 subgroups. *Dendrobium hybrida* MYB proteins clustered in subgroup 9 which is the

same subgroup as MYB proteins that are associated with cell shape development (*AmMIXTA*: Noda, *et al.*, 1994; *AmML1*: Perez-Rodriguez, *et al.*, 2005; *PhMYB1*: Avila, *et al.*, 1993; *AmMYBML2*: Baumann, *et al.*, 2007; *AmMYBML3*: Jaffe, *et al.*, 2007), thus they were hypothesized to play the same role. The group of sequences examined for function in this study included Dh2petalAS2, Dh2MB1, Dh2MB2, Dh2MB4, Dh2miB3, Dh2miB1, Dh2SB1, Dh2MB3 and Dh2MB7. Subsequently, a consensus sequence of this group was used as a guideline in identification of the motifs outside DNA binding domain.

5.3 Characterization and Phylogenetic analysis of the full length *Dendrobium hybrida* R2R3MYB

A full length *Dendrobium hybrida R2R3MYB* cDNA was obtained through the extension of the partial consensus sequence of *Dendrobium hybrida* MYBs that clustered into subgroup 9. Sequence analysis revealed that this *DhMYB* cDNA shares 97% and 73% identity with the *Dendrobium* sp. XMW-2002-1 MYB1 (MYB1) mRNA, complete cds and *Dendrobium crumenatum* MYBML1 mRNA, complete cds, respectively. The function of both genes were not known (Wu, *et al.*, 2003). Alignment of translated full length DhMYB sequence together with those of MYBs in subgroup 9, showed high sequence similarity within R2R3 domain at the N-terminus and shared the same motifs (MGiDPvTHkp--- HmaQWeSARleAEaRLxR$^{E}/_{Q}$SxL; Kranz *et al.*, 1998; Stracke *et al.*, 2001) at the C-terminal domain.

Phylogenetic analysis between translated full length DhMYB and subgroup 9 members, grouped DhMYB together with DwMYB1 and DcMYBML1, both of unknown function, whilst its sister group included AmMIXTA and AmMYBML1 which control cell differentiation in petal and have been reported to activate anthocyanin biosynthetic gene expression and thus influence flower colour (Noda, *et al.*,

1994; Perez-Rodriguez, *et al.*, 2005). This suggested that the deduced DhMYB protein, AmMIXTA and AmMYBML1 share homologous functions.

5.4 Expression profile of the *Dendrobium hybrida MYB* during flower development

The functions of the MYB genes from subgroup 9 were previously studied in *Anthirrhinum majus*, *Arabidopsis thaliana*, *Petunia hybrida* and *Gossypium hirsutum* (Perez-Rodriguez, *et al.*, 2005; Baumann, *et al.*, 2007; Avila, *et al.*, 1993; Machado, *et al.*, 2009). However, no study has been carried out on the expression profile of these genes at different flower developmental stages. We found that expression of *DhMYB* gene increased gradually from bud stage 1 to stage 6 with the highest expression level at stage 6 which corresponds to a bud length of 2.5 – 3.0 cm. The DhMYB cDNA that we isolated seemed to be expressed throughout the development of floral buds. The floral developmental stages in *Dendrobium hybrida* were categorized based on a method previously reported for inflorescence development in *Dendrobium* X Jaquelyn Thomas 'Uniwai Prince' (UH503) (Mudalige-Jayawickrama, *et al.*, 2005).

5.5 Epidermal patterns in *Dendrobium hybrida*

The pattern of epidermis cells in the flower is crucial to understand the function of the petal in the most Angiosperm species (Whitney, *et al.*, 2011). Epidermal cell shape is the first point of contact with both biotic and abiotic environments (Miller, *et al.*, 2011; Whitney, *et al.*, 2011). Flower epidermis pattern was studied in *Lotus japonicus* (Weng, *et al.*, 2011), *Antirrhinum majus* (Baumann, *et al.*, 2007), *Dendrobium* species and hybrids (Mudalige, *et al.*, 2003). It was shown that the dorsal petal, lateral petal and ventral petal of *Lotus japonicus* consisted of four domains, five domains and five domains, respectively (Weng, *et al.*, 2011).

In this project, we have identified various cell types on the adaxial surface of petal, sepal and lip at stage 9 (fully open flower stage) in *Dendrobium hybrida*. Both petal and sepal can be divided into 2 distinct domains based on their epidermal cell shape, whilst lip can be divided into seven domains according to different epidermal cell types from their adaxial surface (Table 4.6). Some epidermal cells are unique to lip, while flat epidermal cell shape is shared among floral parts. However, most of the earlier studies reported that the epidermal patterns in angiosperm only divided to two main types, which are tabular (flat) and papillose (conical) (Mudalige, *et al.*, 2003; Baumann, *et al.*, 2007). Characterization of epidermal cells on petals of legume flowers has been used as a micromorphological marker of different petal identity (Weng, *et al.*, 2011; Ojeda, *et al.*, 2009). Thus, it can be suggested that the characterization epidermal cells on sepal, petal and lip of *Dendrobium hybrida* flowers can also be used as a micromorphological marker.

Our data showed that the *Dendrobium hybrida* lip possesses conical epidermal cell shape when compared with perianth epidermal cell shape. This suggests that conical cell shape in lip could focus more light over a greater part of it's surface thus enhancing colour in lip (Mudalige, *et al.*, 2003; Whitney, *et al.*, 2011; Weng, *et al.*, 2011; Comba, *et al.*, 2000). Moreover, the existence of surface striations solely in lip perhaps function in reducing the surface reflection, thus, enhancing the light absorption (Kay, *et al.*, 1981; Mudalige, *et al.*, 2003).However, in our study, there is no changes in flower colour eventhough dsRNA knockdown of the *DhMYB* gene caused the cell shape changes in lip. In additional, *Dendrobium hybrida* showed less papillose cells from distal end to proximal end. This suggested that this zonation act as guidance to pollinator for landing at more papillose cells distal end due to this papillose cells increase grip (Alcorn, *et al.*, 2012; Glover and Martin, 1998; Whitney, *et al.*, 2011). In *Dendrobium hybrida*, darker lip colour was observed in control flower when compared with other perianth parts. This

suggested that surface striations and different epidermal cell patterns caused darker lip (Figure 3.1) (Mudalige, *et al.*, 2003).

5.6 *DhMYB* cDNA functions in conical cell differentiation

RNAi knock down of the expression of *DhMYB* using *DhMYB* dsRNA was carried out in this study. Interestingly, only conical cell shape in lip was flattened. This revealed that the *Dendrobium hybrida* MYB can only promote conical cell growth in the lip but not in the perianth at stage 9, when the flower opened and at the 100 μg of the crude extracts of bacterially expressed *DhMYB* dsRNA .Hence, this suggested that DhMYB may play a role as an epidermal cell-shape regulator that promotes elongation in the lip epidermis, resulting in conical cells, which may enhance the attractiveness of lip to insect pollinators (Alcorn, *et al.*, 2012; Baumann, *et al.*, 2007). AmMIXTA and AmMYBML1 have been reported to regulate the differentiation of conical-papillate petal epidermal cells and initiate trichome development in antirrhinum (Perez-Rodriguez, *et al.*, 2005; Noda, *et al.*, 1994; Glover and Martin, 1998; Baumann, *et al.*, 2007). These results revealed that DhMYB may not be orthologous to its sister group, AmMIXTA and AmMYBML1, although the phylogeny analysis suggests it to be closely related to them.

5.7 Crude extracts of bacterially expressed *DhMYB* dsRNA can be used to study gene function

Previous studies have used the transgenic expression of RNAs for functional study of subgroup 9 *R2R3 MY*B genes in Antirrhinum, tobacco, Petunia and Thalictrum (Di Stilio, *et al.*, 2009; Baumann, *et al.*, 2007; Perez-Rodriguez, *et al.*, 2005). In our study, exogenous dsRNA derived from partial *DhMYB* sequences was used as an initiator to knock down the gene expression of *DhMYB* in *Dendrobium hybrida* by directly delivering dsRNA to leaf cells through mechanical inoculation (Waterhouse, *et al.*,

1998;Tenllado and Diaz-Ruiz, 2001; Tenllado, *et al.*, 2003; Sun, *et al.*, 2010). Our approach differs from strategies based on transgenic expression of RNAs but still relies on PTGS to achieve RNA silencing of the target gene (Tenllado, *et al.*, 2003; Ji and Chen, 2012). In addition, this approach was successfully and widely used to study viral resistance for example in tobacco, rice and maize (Tenllado, *et al.*, 2003; Gan, *et al.*, 2010; Sun, *et al.*, 2010). Our data also revealed that the expression level of *DhMYB* was lower in the treated *Dendrobium hybrida* when compared with untreated control *Dendrobium hybrida*.

Mechanical inoculation of RNA using bacterially expressed genes is a protocol that is simple and quick when compared with using either the *in vitro* synthesis of large amounts of RNA or the introduction of transgene into plants (Tenllado and Diaz-Ruiz, 2001; Tenllado, *et al.*, 2003; Yin, *et al.*, 2009; Gan, *et al.*, 2010; Sun, *et al.*, 2010). In the current study, this approach was found to successfully reduce transcript levels in *Dendrobium hybrida* and result in a phenotype that can be informative about the gene function.

Chapter 6.0 Conclusion

In this study, 37 partial and 1 full length *Dendrobium hybrida* R2R3 MYB cDNA were successfully cloned and sequenced. Phylogenetic analysis showed that the translated MYB protein (DhMYB) sequences were clustered in the subgroup 9 with MYB proteins that are well known in cell shape development (AmMIXTA, AmML1, PhMYB1 and AmMYBML1). Quantitative RT-qPCR evaluation of *DhMYB* was analyzed during flower development and showed that the transcript levels of *DhMYB* increased gradually from bud stage 1 to stage 6 and decreased at stage 7 during normal flower development. Functional study of a full length *DhMYB* cDNA by RNAi knockdown suggests that *DhMYB* cDNA may plays a role in lip cell morphogenesis in orchid, but may not play a role in colour development. This study also demonstrated that mechanical inoculation of orchid flower buds with dsRNA using bacterially expressed genes is a simple, fast, safe and inexpensive protocol to study flower gene function.

Future study

From this study, we can deduce that *DhMYB* cDNA may play a role in lip cell morphogenesis in *Dendrobium hybrida*. However, some questions remain unanswered. For example, which basic helix loop helix (*bHLH*) and WD40-repeat protein(s) complex with DhMYB 1 to control cell shape morphogenesis of flowers? What is the stability of sRNA during the experiment? Does MYB play a role in other pathways e.g. can that be observed if a stronger knock down is achieved? What is the consequence if *Dendrobium hybrida* is subjected to different time frames for MYB expression knock down? What does this gene do in other tissues (e.g. leaf, root)? What are other possible gene targets to alter *Dendrobium hybrida* flower colour?

In order to answer these questions, further experiments need to be done. A yeast two hybrid system can be used to study the interaction of the basic helix loop helix (*bHLH*) and WD40-repeat protein with DhMYB protein. RNAi mediated by an exogenous dsRNA approach can be used to study the function of identified bHLH and WD40 gene. RNAi of DhMYB and bHLH and WD40 candidates can be carried out by a range of dsRNA concentration and inoculation time point to observe the changes of flower colour and other genes such as pigment biosynthesis could also be tested. These experiments could be taken up to understand the interaction between MYB, bHLH and WD40, to investigate the optimum parameters for RNAi using mechanical treatment approach and to alter *Dendrobium hybrida* flower colour.

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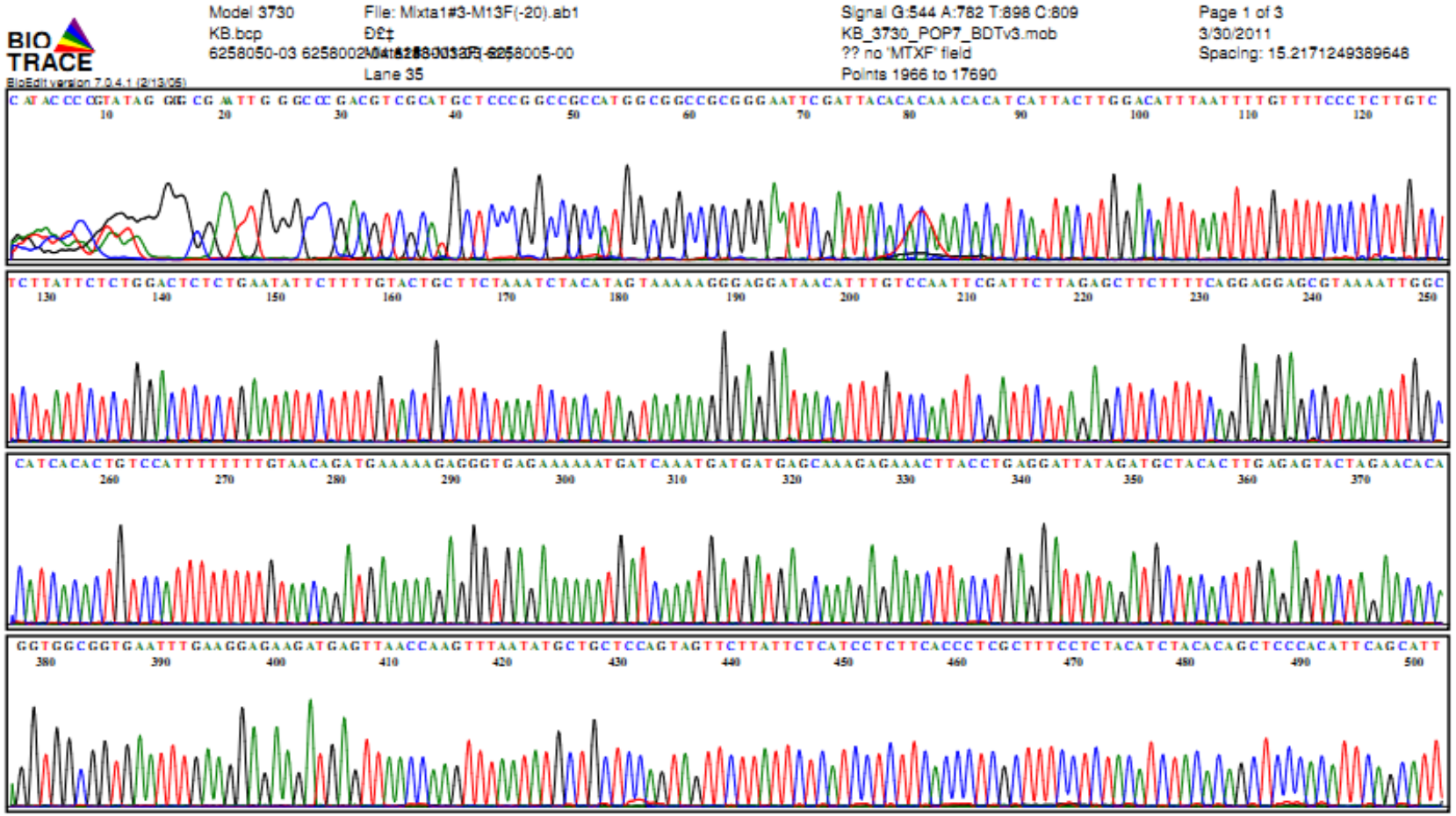
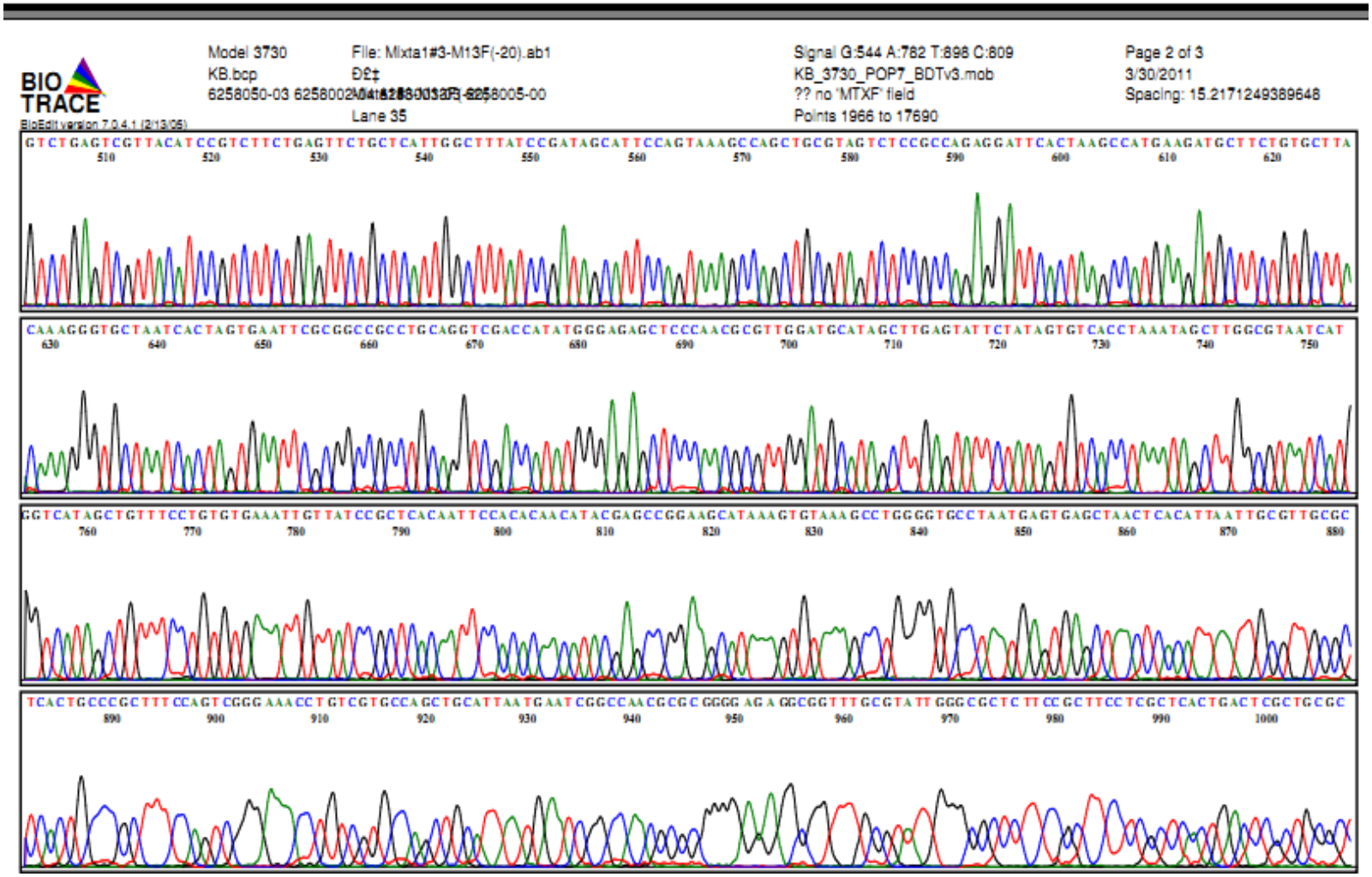
APPENDIXES

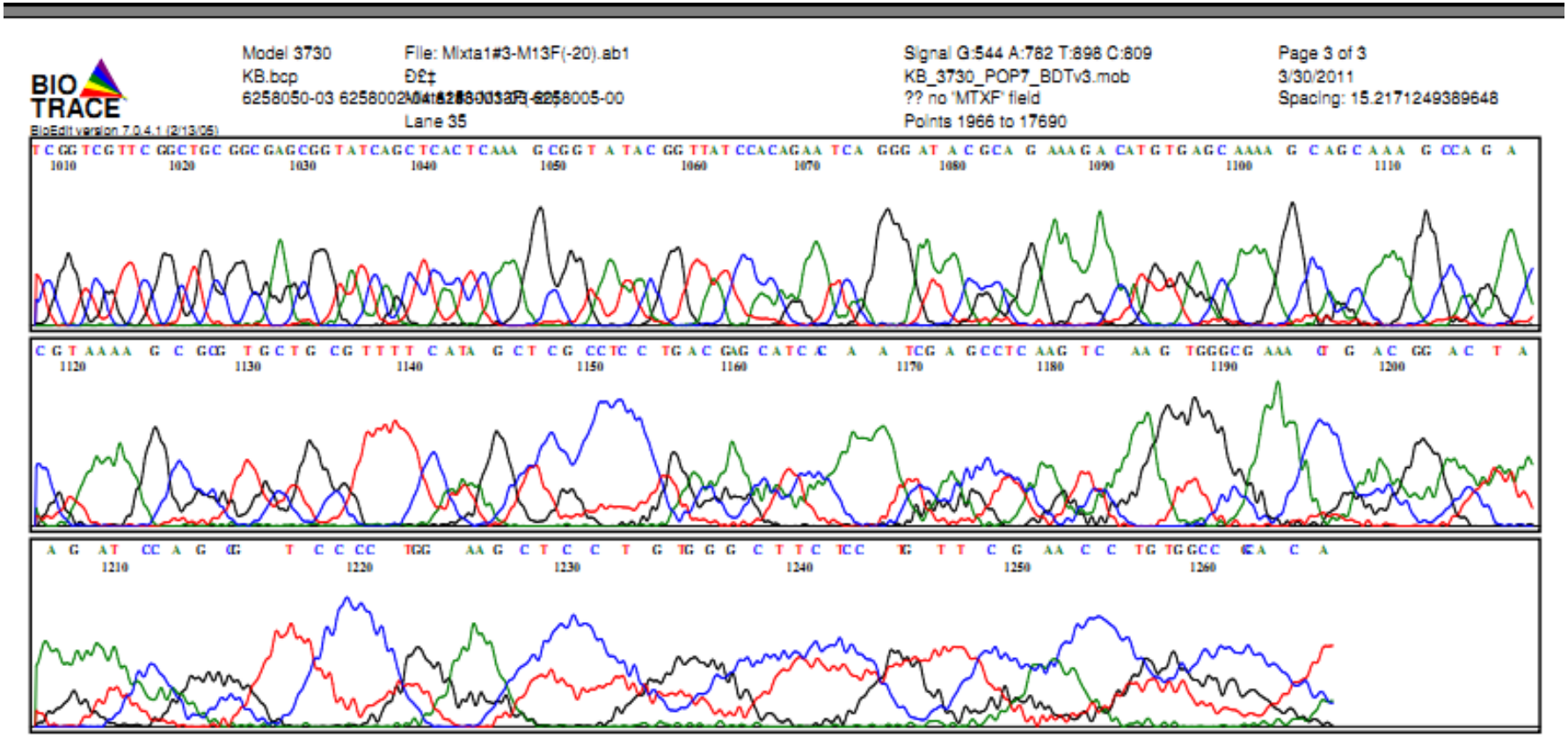
Appendix 1Chromatograms of *DhMYB* 3'RACE



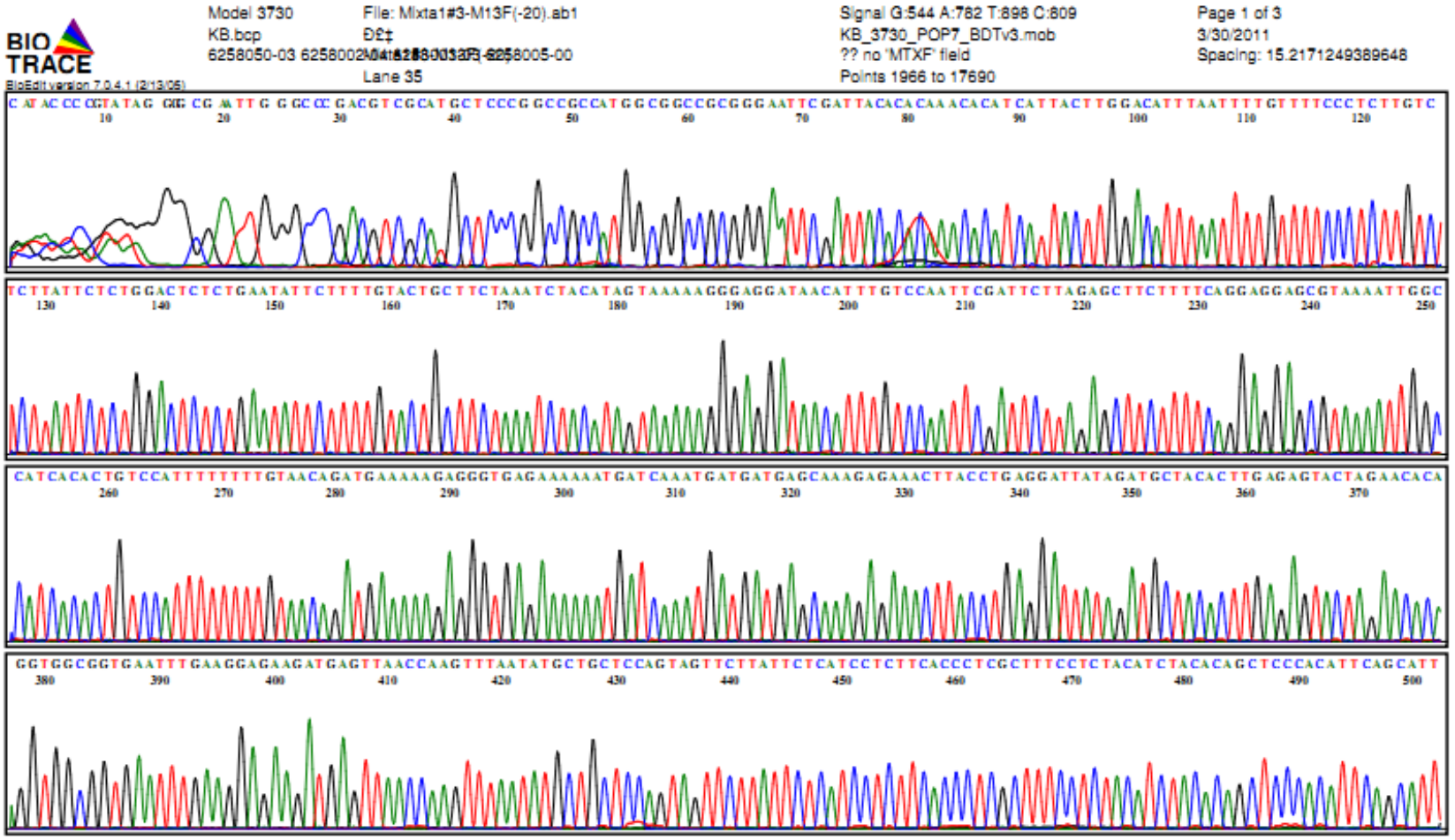
Appendix 1 continued Chromatograms of *DhMYB* 3'RACE



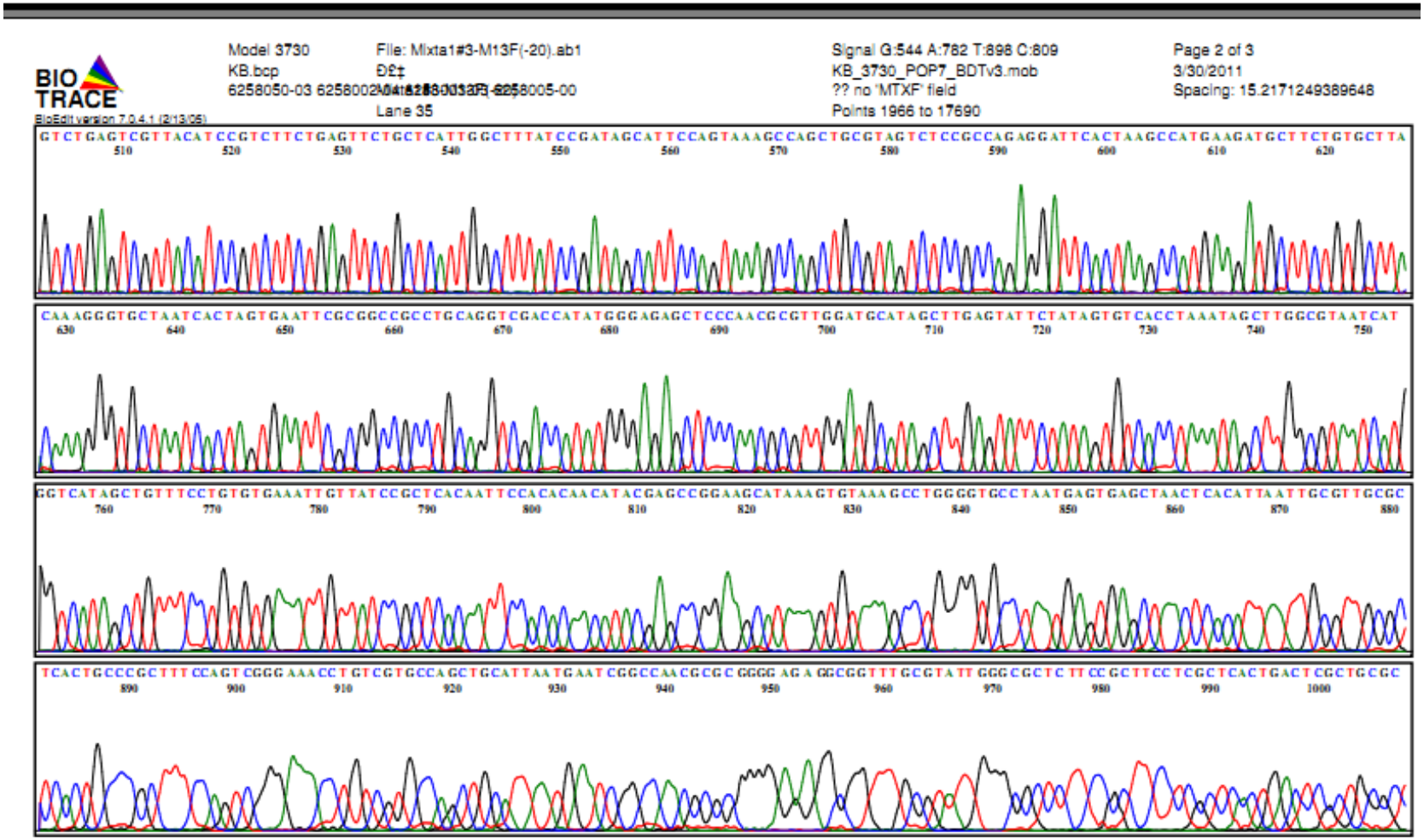
Appendix 1 continued Chromatograms of *DhMYB* 3'RACE



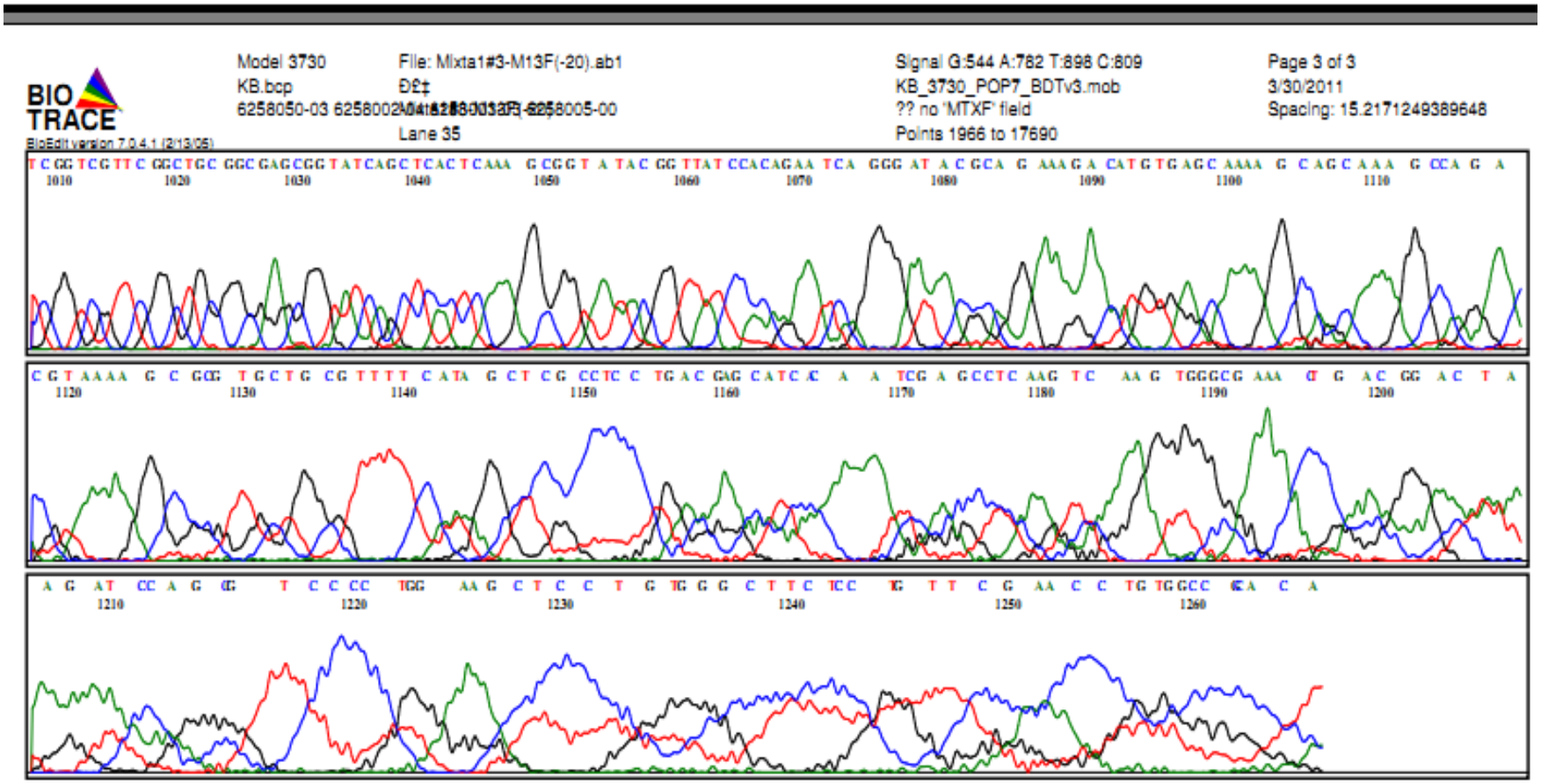
Appendix 2 Chromatograms of *DhMYB* 5'RACE



Appendix 2 continued Chromatograms of *DhMYB* 5’RACE



Appendix 2 continued Chromatograms of *DhMYB* 5’RACE



Appendix 3

Appendix 3 (continued)

Appendix 3 (continued)

Appendix 4: The relationship between bud or flower stage and mean days taken to reach each stage from emerged bud

Growth stage	1	2	3	4	5	6	7	8	9
No. 1	5	9	13	16	20	24	27	28	30
2	7	13	17	21	24	29	32	33	35
3	5	10	14	18	21	25	28	29	31
4	6	11	15	18	21	25	28	29	31
5	7	12	16	19	22	25	28	29	31
6	5	11	15	18	21	24	26	27	29
7	5	11	15	18	22	25	28	29	30
8	6	12	16	20	24	28	31	32	33
9	5	11	14	17	20	23	26	27	28
10	5	10	14	17	20	23	28	32	33
11	6	13	17	19	21	25	29	30	32
12	6	12	16	19	22	25	28	29	31
13	5	10	14	18	22	24	29	30	31
14	5	11	15	18	21	24	28	29	31
15	5	12	15	18	22	25	29	30	31
16	5	11	15	18	21	24	28	32	33
17	5	10	14	17	19	22	26	27	29
18	6	12	16	20	22	26	30	31	32
19	6	11	15	18	21	24	27	28	30
20	6	11	15	17	19	22	27	30	32
21	5	12	16	19	22	25	29	30	32
22	7	13	18	22	26	29	33	34	36
23	7	14	17	20	22	29	32	33	35
24	7	14	18	21	24	29	31	32	34
25	5	10	15	18	22	25	29	33	35
26	5	11	15	17	19	22	26	31	33
27	5	11	16	18	22	27	31	32	33

	28	5	12	17	20	23	27	31	32	34
	29	5	11	15	18	21	25	30	31	33
	30	5	12	16	19	23	26	29	30	32
Mean (Day)		6	11	15	19	22	25	29	30	32
SD		0.77	1.19	1.22	1.38	1.58	2.05	1.91	1.93	1.94
SE		0.14	0.22	0.22	0.25	0.29	0.37	0.35	0.35	0.35

Appendix 5: Relative efficiency of target (*DhMYB* cDNA) and endogenous control (*ß-actin* cDNA)

Total RNA (ng)	*DhMYB* cDNA average C_T	*ß-actin* cDNA average C_T	ΔC_T *DhMYB* - *ß-actin*
1	20.13 ± 0.04	17.38 ± 0.14	2.75 ± 0.15
0.3	22.39 ± 0.08	19.47 ± 0.09	2.92 ± 0.12
-0.4	25.05 ± 0.02	22.00 ± 0.10	3.05 ± 0.1
-1.1	27.37 ± 0.06	24.47 ± 0.04	2.9 ± 0.07

Appendix 6: Relative quantification of *DhMYB* cDNA in bud stage 1 to 7 tissues. Transcript levels were normalized relatively to *ß-actin* cDNA (endogenous control).

Tissue (Bud stage)	*DhMYB* cDNA average C_T	*ß-actin* cDNA average C_T	[a]ΔC_T *DhMYB* - *ß-actin*	[b]$\Delta\Delta C_T$ ΔC_T -ΔC_T, bud stage 1	[c]Mean $\Delta\Delta C_T$	[d]DhMYB $cDNA_N$ relative to bud stage 1	[e]log DhMYB $cDNA_N$ relative to bud stage 1
1 (R1)	24.97 + 0.12	18.67 + 0.12	6.3 ± 0.17	0 ± 0.17	0 ± 0.17	1 0.89 - 1.13	0 -0.12 – 0.12
2 (R1)	25.2 ± 0.08	20.53 ± 0.14	4.67 ± 0.16	-1.63 ± 0.16	-1.62 ± 0.16	3.07 2.75 - 3.43	1.12 1.01 – 1.23
3 (R1)	23.51 + 0.09	20.34 ± 0.08	3.17 ± 0.12	-3.13 ± 0.12	-3.24 ± 0.12	9.45 8.69 – 10.27	2.25 2.16 – 2.33
4 (R1)	24.35 + 0.06	21.7 + 0.15	2.65 ± 0.16	-3.65 ± 0.16	-3.43 ± 0.16	10.78 9.65 -12.04	2.38 2.27 – 2.49
5 (R1)	20.29 + 0.04	18.06 + 0.13	2.23 ± 0.14	-4.07 ± 0.14	-4.15 ± 0.14	17.75 16.11 - 19.56	2.88 2.78 – 2.97
6 (R1)	23.36 + 0.19	21.5 + 0.14	1.86 ± 0.24	-4.44 ± 0.24	-4.41 ± 0.24	21.26 18.00 - 25.11	3.06 2.89 – 3.22
7 (R1)	23.36 ± 0.02	20.98 ± 0.16	2.38 ± 0.16	-3.92 ± 0.16	-3.82 ± 0.26	14.12 11.79 - 16.91	2.65 2.47 – 2.83
1 (R2)	26.21 + 0.14	19.84 + 0.13	6.37 ± 0.19	0 ± 0.19			
2 (R2)	26.45 ± 0.1	21.71 ± 0.05	4.74 ± 0.11	-1.63 ± 0.11			
3 (R2)	24.66 ± 0.02	21.74 ± 0.11	2.92 ± 0.11	-3.45 ± 0.11			
4 (R2)	22.34 + 0.24	19.38 + 0.08	2.96 ± 0.25	-3.41 ± 0.25			
5 (R2)	24.16 + 0.12	22.06 + 0.26	2.1 ± 0.29	-4.27 ± 0.29			

6 (R2)	24.13 + 0.18	22.30 + 0.20	1.83 ± 0.27	-4.54 ± 0.27			
7 (R2)	23.17 ± 0.02	20.7 ± 0.17	2.47 ± 0.17	-3.9 ± 0.08			
1 (R3)	29.22 + 0.21	23.1 + 0.12	6.12 ± 0.24	0 ± 0.24			
2 (R3)	24.95 ± 0.05	20.42 ± 0.08	4.53 ± 0.09	-1.59 ± 0.09			
3 (R3)	23.69 ± 0.13	20.71 ± 0.07	2.98 ± 0.15	-3.14 ± 0.15			
4 (R3)	23.42 ± 0.14	20.52 ± 0.15	2.9 ± 0.21	-3.22 ± 0.21			
5 (R3)	21.44 + 0.13	19.43 + 0.38	2.01 ± 0.4	-4.11 ± 0.4			
6 (R3)	21.49 ± 0.09	19.61 ± 0.28	1.88 ± 0.29	-4.24 ± 0.29			
7 (R3)	23.66 ± 0.07	21.17 ± 0.03	2.49 ± 0.08	-3.63 ± 0.08			

[a]The ΔC_T value is determined by subtracting the average *ß-actin* cDNA C_T value from the average *DhMYB* cDNA C_T value. The standard deviation of the difference is calculated from the standard deviations of the *DhMYB* cDNA and *DhMYB* cDNA *ß-actin* cDNA values.
[b]The calculation of $\Delta\Delta C_T$ involves subtraction by the ΔC_T of bud stage 2. This is subtraction of an arbitrary constant, so the standard deviation of $\Delta\Delta C_T$
is the same as the standard deviation of the ΔC_T value.
[c]Mean of the $\Delta\Delta C_T$ from three biological replicates of each bud stage.
[d]The range given for *DhMYB* cDNA relative to bud stage 2 is determined by evaluating the expression: $2^{-\Delta\Delta C_T}$
[e]Logarithm of relative quantification.

Appendix 7

Appendix 7

Appendix 7

Appendix 8: Comparison of appearance of buds at after treatment with dsRNA

Samples	Image
Control 1	
Control 2	
Control 3	
Treated with DhMYB dsRNA 1	
Treated with DhMYB dsRNA 2	
Treated with DhMYB dsRNA 3	 Left panel: control Right panel: Treated with DhMYB dsRNA