ISOLATION AND CHARACTERIZATION OF cDNA ENCODING DIHYDROFLAVONOL 4-REDUCTASE GENE FROM ORCHID *DENDROBIUM* VARIETY SONIA17.

SARITHA, V. S.

Thesis submitted in partial fulfillment of the requirement For the degree of

Master of Science in Agriculture

Faculty of Agriculture
Kerala Agricultural University, Thrissur

2009

Department of Plant Biotechnology
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM 695522

DECLARATION

I hereby declare that this thesis entitled **"Isolation and characterization of cDNA encoding dihydroflavonol 4-reductase gene from Orchid *Dendrobium* variety Sonia 17"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

Vellayani
20-01-2009

SARITHA, V. S.
(2006-11-115)

CERTIFICATE

Certified that this thesis entitled **"Isolation and characterization of cDNA encoding dihydroflavonol 4-reductase gene from Orchid *Dendrobium* variety Sonia 17"** is a record of research work done independently by Ms. Saritha,V. S. (2006-11-115) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

Vellayani
20-01- 2009

Dr. K. B. Soni
(Chairman, Advisory Committee)
Associate Professor
Department of Plant Biotechnology,
College of Agriculture, Vellayani,
Thiruvananthapuram-695522

APPROVED BY

Chairman:

Dr. K. B. SONI
Associate Professor
Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram – 695 522.

Members:

Dr. K. RAJMOHAN
Professor and Head
Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram – 695 522.

18/7/2009

Dr. SWAPNA ALEX
Associate Professor
Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram – 695 522.

18/7/2009

Dr. R. V. MANJU
Assistant Professor (SS)
Department of Plant Physiology
College of Agriculture, Vellayani
Thiruvananthapuram – 695 522.

18/7/09

EXTERNAL EXAMINER

Dr. V. KRISHNASAMY
Professor and Head
Dept. Plant Molecular Biology and Biotechnology
Tamil Nadu Agricultural University
Coimbatore-641 003

18.7.09

Dedicated to

My Beloved Parents and Teachers

ACKNOWLEDGEMENT

"In every wisp of breath, thought, word and deed, let me be with my Beloved God"

My heartfelt thanks and gratitude to my Chairperson Dr. K. B. Soni, for her inestimable and meticulous guidance, sustained interest, constant encouragement, constructive ideas, continuous help and critical suggestions shown throughout my studies.

I am greatful to Dr. K. Rajmohan, Professor and Head, Department of Plant Biotechnology, for his valuable guidance, encouragement, support and critical suggestions during the entire of my thesis work.

I wish to place my sincere thanks to Dr. Swapna Alex, Associate professor, Department of Plant Biotechnology, for her help throughout the study.

My most sincere thanks to Dr. R. V. Manju, Associate professor, Department of Plant Physiology, for her support and help.

I greatfully acknowledge Dr. Roy Stephen, Associate professor, Department of Plant Physiology, for his constant encouragement and friendly approach.

I am shortage of words to express my heartfelt thanks to Anu chechi, for the love and care that she given for me till this time.

I am deeply thankful to Vimarsh, for his support, encouraging words, and timely help will never be forgotten.

My heartfelt thanks to Asha, Smitha Bhasi and Lekshmi for their unsolicited help and pleasant company.

I am also thankful to Soni, Vinitha, Akhila, Anjana, Nanditha, Rakhi, Sajeena for their immense help.

It is great pleasure to express my gratitude to Vidya, Bhavya, Divya, Prabhu, Selvaraju, Kumanan, Renjini, Revathi, Arpitha and Thankamony chechi for their friendship and help.

My list of expressing gratitude will be incomplete unless I offer my best regards and wishes to Mr. Vincent, Anandakalakendram, Kanjiramkulam for providing me with Dendrobium Sonia 17 flower buds.

I am shortage of words to express my thanks and gratitude to my parents, my father for giving me courage and determination to finish my studies despite many obstacles, my mother who always believed in me and my most wonderful brothers and sister in law for their love and support throughout the long years of my education.

From the depth of my heart I thank each and every member of my family, my aunty, pappa, Rajeev chettan who helped me a lot in many ways and the support they given will never be forgotten.

Thanks all.....

Saritha, V. S.

CONTENTS

No	Chapter	Page No
1	INTRODUCTION	1-2
2	REVIEW OF LITERATURE	3-37
3	MATERIALS AND METHODS	38-52
4	RESULTS	53-56
5	DISCUSSION	57-61
6	SUMMARY	62-63
7	REFERENCES	64-89
	APPENDIX	90-92
	ABSTRACT	

LIST OF TABLES

Table No.	Title	Page. No.
1	Details of *dfr* gene sequences used for designing gene specific primer D	54-55
2	Details of primers designed	51-55

LIST OF FIGURES

Sl. No	Title	Between Pages
1	Basic carbon skeleton of the anthocyanin molecule	7-9
2	Anthocyanin and flavonol biosynthesis pathway	9-10
3	Chemical reaction catalyzed by dihydroflavonol 4-reductase gene	14-16
4	pGEM-T Easy Vector circle map sequence reference points	48-49
5	The promoter and multiple cloning sequence of the pGEM-T Easy Vector	48-49
6	Input form for Clustal X 1.81 multiple alignment programme for the Primer D	53-54
7	BLASTN result for the Primer set D	53-54

LIST OF PLATES

Plate. No.	Title	Between Pages
1	Flower buds of *Dendrobium* Sonia 17 orchid showing different stages	54-55
2	Total RNA extracted with RRII extraction protocol	54-55
3	Total RNA extracted with TRIZOL reagent	54-55
4	cDNA amplified using dihydroflavonol 4-reductase specific primer in orchid *Dendrobium* Sonia 17	55-56
5	Transformed *E. Coli* colonies	55-56
6	PCR amplification of plasmid DNA using gene specific primer A	55-56

LIST OF APPENDICES

Sl. No.	Appendices
I	1X TAE buffer composition
II	S. O. C. medium components
III	Reagents for plasmid DNA isolation

LIST OF ABBREVIATIONS

A	Adenine
Acc. No.	Accession number
AMV	Avian myeloblastosis virus
ANS	Anthocyanin synthase
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Cytisine
CHS	Chalcone synthase
CTAB	Cetyl trimethyl ammonium bromide
cDNA	Complementary DNA
CoA	Co enzyme A
DEPC	Diethyl pyrocarbonate
DFR	Dihydro flavonol 4-reductase
DHK	Dihydrokaempferol
DHQ	Dihydroquercetin
DHM	Dihydromyricetin
DNA	Deoxy ribonucleic acid
dNTP	Deoxynucleotide triphosphate
d(T)	Deoxy thymydilic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
FASTA	Fast Alignment
F3'5'H	Flavonoid 3'5'- hydroxylase
G	Guanine
IPTG	Isopropyl-beta-thio-galactopyranoside
LB	Luria bertaini broth
LiCl	Lithium chloride

M	Molar
mM	Millimolar
M-MuLV	Moloney Murine Leukemia Virus
mRNA	Messenger RNA
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
ng	Nanogram
PCR	Polymerase Chain Reaction
Poly (A)	Poly adenosine
PVP	Polyvinyl pyrrollidone
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse Transcription -PCR
SDS	Sodium dodecyl sulphate
SDH	Shikimate dehydrogenate
T	Thymine
TAE	Tris- acetate EDTA
Taq	*Thermus aquaticus*
TE	Tris-HCL-EDTA
Tris	Tris (hydroxyl methyl) aminomethane
Tris-Hcl	Tris (hydroxyl methyl) aminomethane hydrochloride
U	Uracil
X-Gal	5-Bromo- Chloro-3-Indolyl-B-D-Galactopyranoside
µl	Microlitre
µg	Microgram

Introduction

1. INTRODUCTION

Orchids belong to the family orchidaceae which comprise the largest family of flowering plants. Taxonomically they represent the most evolved family among the monocotyledons, with 700-800 genera, 25,000- 30,000 species and a lakh man made hybrids (Singh, 1986). Orchid cut flower industry in the world today is a highly developed trade for local and export markets. Development of new hybrids and commercial cultivation of orchids have become a lucrative industry.

Dendrobium is one of the most important cut flower orchid currently enjoying very high popularity in markets (Lekha Rani, 2002). It is the second largest genera of orchid family with more than 1000 species (Dressler, 1990). *Dendrobium* hybrids are the most suitable and popular among the commercial orchids grown throughout the country. *Dendrobium* variety Sonia (*D. caesae x D. tomie Drake*) is well known for its attractive flowers.

Orchid flowers show an incredible range of diversity in size, shape, colour, structure, number and fragrance. Among the different factors which determine the market value, flower colour is one of the important traits. Due to the ever-changing demand in the market, there is a need to continuously produce varieties with newer traits. Altered and improved flowered varieties fetches very high price in the market. Plant breeders have been attempting to introduce novel colours in orchids. Traditional breeding techniques are, however limited by the absence of key pigment biosynthesis genes in a particular species gene pool. Genetic engineering techniques are used as an alternative to traditional breeding to create novelty by altering specific traits.

Modification of flower colour *via* genetic engineering has generally focused on metabolic engineering of the anthocyanin biosynthetic pathway. Anthocyanin biosynthetic pathway is very complex and involves large number of structural and regulatory genes. Dihydroflavonol 4-reduactase is one of the key biosynthetic genes which regulate the flower colour. In addition to the genetic factors, vacuolar pH, light

and temperature also have an effect on anthocyanin biosynthesis. Most of the genes coming under this pathway have been cloned from many plant species and the knowledge about these genes are widely used to create novel flower colours and few of them are already available in the market such as blue/violet carnations and rose. Although molecular characterization of orchids is increasing (Kuehnle, 1997 and Mudaligae and Kuehnle, 2004) it still lags behind other flower crops.

The unusual colour combination in *Dendrobium* species and hybrids make them attractive for chemical and molecular genetic analysis. *Dendrobium* flower colours are in range of white, pink, red, blue, purple to yellow. However, some colours are missing from *Dendrobium* flower colour spectrum. For altering flower colour in *Dendrobium* orchids through metabolic engineering isolation and characterization of key pigment biosynthetic gene are essential. So far no studies have been reported in *Dendrobium* Sonia 17 orchids in this regard.

In this context the present work was focused on the isolation and characterization of the gene encoding key anthocyanin biosynthetic enzyme, namely dihydroflavonol 4-reductase from flower buds of orchid *Dendrobium* variety Sonia 17 using Reverse transcription-PCR. This information will supplement the genomic databases of *Dendrobium* orchids which will enable the genetic improvement of this flower crop.

Review of literature

2. REVIEW OF LITERATURE

Major floral diversity distinguishes orchidaceae as one of the largest and most evolved families among flowering plants. Unique flower shapes, fragrance, colours and keeping quality also render several orchid genera highly priced as commercial cut flowers and blooming potted plants. Hybrids within the genus *Dendrobium* comprise one of the top traded groups of orchids in the domestic (Lekha Rani, 2002) and international market. *Dendrobium* flowers are usually white, mauve, pink, red, blue, purple and yellow (Mercy and Dale, 1997). Classical breeding techniques are given rise to many commercially successful hybrids with attractive flower colour. However some colours are missing from *Dendrobium* flower colour spectrum (Kuehnle et al., 1997). Genetic modification of pigment biosynthesis through biotechnology is becoming an integral part of breeding new colours in ornamentals (Tanaka et al., 2005).

Flower colour is mainly due to flavonoids and their coloured class of compounds - anthocyanins (Harborne and Williams, 2000). The versatile chemical structure of anthocyanins, their co-existence with other flavonoids (flavones and flavonols), the presence of metal ions, vacuole pH, as well as environmental factors such as light and temperature also affect the final shades of flower colour (Tanaka et al., 2005 and Tanaka and Brugliera, 2006).

Metabolic engineering of flavonoid pathway is very well understood and most of the genes involved in this pathway are elucidated from many plant species (Grotewold, 2006 and Tanaka et al., 2005). Modification of this pathway has been achieved in different crops species by different approaches such as incorporation of key biosynthetic genes (orange petunia), antisense or sense suppression of genes

(pale coloured torenia), RNA interference (RNAi) technology (pink torenia) or by transposon mediated colour engineering (variegated flower colour in morning glory) (Tanaka et al., 2005; Tanaka, 2006 and Nakatsuka, 2007).

For metabolic engineering in *Dendrobium* orchids a detailed knowledge about its biosynthetic pathway and key flower colour genes are essential. As an initiative to this, the present study was taken up. The studies conducted in other ornamentals based on these aspects are presented in this chapter.

2.1 MOLECULAR BASIS OF FLOWER COLOUR

Flower colour is a very complex characteristic that involves the chemical interaction of two different types of flavonoids - the anthocyanins and co-pigments (Kondo et al., 1992). The inheritance of anthocyanin pigmentation is controlled by multiple independent genes. The anthocyanin biosynthetic pathway is very complex with large number of structural genes, that control single biosynthetic steps of the various flavonoid classes or steps of flavonoid modification and regulatory genes that switch on or off the whole pathway or part of it that influence flavonoid concentration or that lead to pattern formation (Forkmann and Martens, 2001). Any alteration in these genes can affect pigmentation (Jaakola, 2003).

2.1.1 FLAVONOIDS

Flavonoids, a large family of low molecular weight polyphenolic secondary metabolites, are widespread throughout the plant kingdom (Koes et al., 1994). Water-soluble flavonoids are the most common pigments in flowers which are responsible for a range of colours from yellow - red to violet - blue. The flavonoids are classified into dozens of groups depending on their structure. Among these

groups, chalcones, aurones, anthocyanins, flavones, and flavonols are the major compounds contributing to flower colour (Goto and Kondo, 1991). Flavones and flavonols are almost colourless to the human eye but they may form complexes with anthocyanins. This complex formation causes a bathochromic shift (bluer and deeper colour) to the anthocyanin molecules in the complex (Harborne, 1994)

2.1.1.1 Flavonoid functions

In nature flavonoids are involved in a wide range of functions such as pigmentation of flowers, fruits and seeds, protection against ultraviolet light, plant defense against pathogenic micro organisms, signal molecules in plant-microbe interactions, protection of leaf cells from photo-oxidative damage and as antioxidant that scavenges radicals that are formed from various biotic and abiotic processes (Bohm, 1998 and Harborne and Williams, 2000)

2.1.1.2 Flavonoid modifications

At every point of flavonoid biosynthesis, flavonoids can either be converted into other flavonoid classes *via* the core biosynthetic pathway or can be derivatized to give the distinct compounds found in plants (McIntosh and Mansell, 1990). Flavonoids have several hydroxyl groups that can be extensively modified. Many enzymes have been described that catalyze hydroxylation, methylation, glycosylation, acylation and a number of other reactions on flavonoids (Heller and Forkmann, 1993). These modifications account for the overwhelming diversity of flavonoid metabolites observed in nature (Heller and Forkmann, 1993 and Harborne, 1994).

Transferring a sugar moiety to flavonoid substrates, known as glycosylation, is the most common flavonoid modification process (Heller and Forkmann, 1993). Several roles have been postulated for glycosylation of various compounds in plants. It allows solubilization of compounds in water that provides access to the movement of essential nutrients and organic materials within the plant system (Hrazdina, 1988) and it also acts to detoxify harmful metabolites (Kreuz et al., 1996), and can regulate the action of functional compounds (Szerszen et al., 1994). Due to these characteristics, most flavonoids that occur naturally are found in a glycosylated form. The sugar can either attach directly to the flavonoid skeleton by forming a carbon-to-carbon glycosyl (C-glycosyl) compound, or it can attach *via* a hydroxyl group on the flavonoid, forming an O-glycoside (Heller and Forkmann, 1993). A commonly used sugar in the process of glycosylation is glucose and the reaction is catalyzed by UDP-glucose: 3-oxyglucosyltransferase to produce a stable, soluble compound (Poulton, 1990).

2.1.2 ANTHOCYANINS

Anthocyanins, a particular class of flavonoids that accumulate in the vacuole are major constituents of orange, red, violet, and blue flowers (Winkel- Shirley, 2001). Three different classes of anthocyanidins are responsible for the primary shade of the flower colour; pelargonidin (orange to brick red), cyanidin (red to pink), and delphinidin (purple to blue). The versatile chemical structure of anthocyanins and their co-existence with other flavonoids (flavones and flavonols) further increases the range of flower colour seen in nature (Hanumappa et al., 2007).

2.1.2.1 Flower pigment distribution and spacial location of anthocyanin

Key components of colour in flowers of vast majority of higher plants are flavonoids and anthocyanins located in vacuoles, and, carotenoids and chlorophylls, located in plastids (Strack and Wray, 1993 and Mol et al., 1998). Flowers acquire their characteristic hue due to these pigments in combination with other chemical and physical factors. The physical factors encompass spatial location of pigments and the optical properties of petal epidermal cells (Kay et al., 1981 and Mol et al., 1998). Anthocyanins are confined to a single layer of cells, either to the epidermal or to the sub epidermal layer (Kay et al., 1981 and Christen and Hansen, 1998) in petals and sepals of all light coloured flowers. In contrast, anthocyanins are present either in two cell layers (epidermal and sub epidermal layer), or in many cell layers of epidermal and mesophyll, in all more intensely coloured flowers. These physical factors influence the role of the plant epidermis in pollinator attraction and our perception of flower colour and visual texture (Noda et al., 1994; Gorton and Vogelmann, 1996 and Glover, 2000).

2.1.2.2 Anthocyanin skeleton

The basic skeleton of anthocyanin molecules consists of two aromatic rings (A&B) with a heterocyclic (C) ring in the middle. The number of OH groups attached to the B ring and their methylation status influence the colour directly (Stafford, 1990). The hydroxyl group on position 3 is glycosylated by rhamnose or glucose in the stable anthocyanin molecule. According to the modifications of the central C-ring they can be divided in different structural classes like flavanones, isoflavones, flavones, flavonols, and anthocyanins. Vacillations and methylation of the basic skeleton provide an enormous array of colours found in nature (Koes et al., 1994).

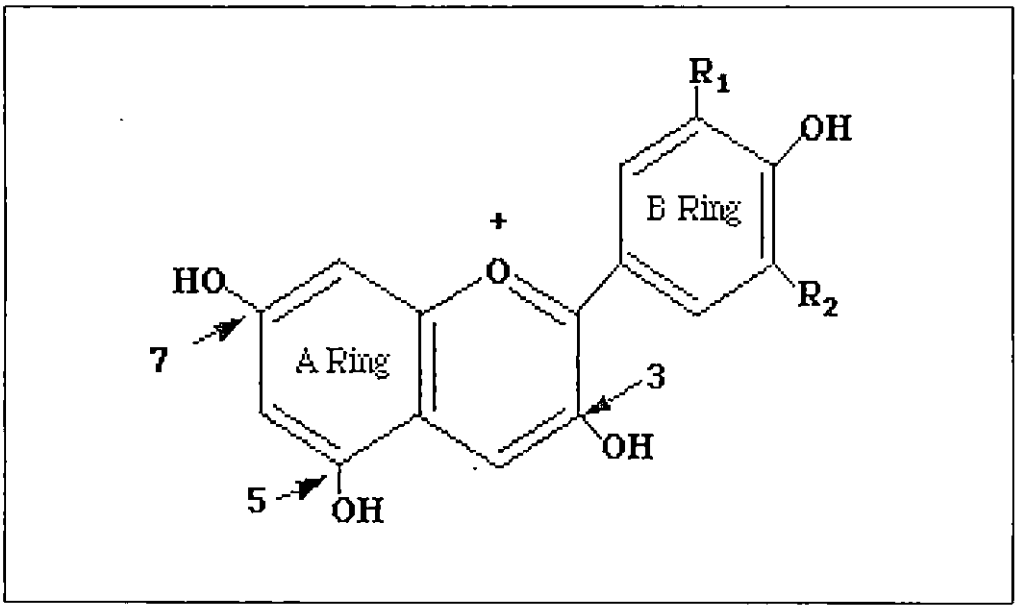

Figure 1. Basic carbon skeleton of the anthocyanin molecule

2.1.2.3 Anthocyanin biosynthetic pathway

Detailed studies conducted by Charrier et al. (1995) and Bernhardt et al. (1998) on the biochemical and molecular aspects of anthocyanin biosynthetic pathway made the understanding of the pathway easier. The anthocyanin biosynthetic pathway is well established and all structural genes leading to anthocyanins in the pathway have been cloned from many plants (Holton and Cornish, 1995 and Winkel-Shirley, 2001).

The precursors of the synthesis of most flavonoids are malonyl-CoA and p-coumaroyl-CoA, which are derived from carbohydrate metabolism and phenylpropanoid pathway, respectively (Forkmann and Heller, 1999). The biosynthesis of flavonoids is initiated by the enzymatic step catalyzed by chalcone synthase (CHS), resulting in the yellow coloured chalcone. Chalcone is isomerised to a flavanone by the enzyme chalcone flavanone isomerase (CHI). From these

central intermediates, the pathway diverges in to several side branches, each resulting in a different class of flavonoids. Flavanone 3-hydroxylase (F3H) catalyzes the steriospecific 3β- hydroxylation of (2S) - flavanones to dihydroflavonols. For the biosynthesis of anthocyanins, dihydroflavonol 4-reductase (DFR) catalyzes the reduction of dihydroflavonols to flavan-3, 4-diols (leucoanthocyanins), which are converted to anthocyanidins by anthocyanidin synthase (ANS). The formation of glucosides is catalyzed by UDP glucose-flavonoid 3- oxy-glucosyl transferases (UFGT), which stabilizes the anthocyanidins by 3-oxy-glucosylation (Harborne, 1994 and Bohm, 1998).

A diagrammatic representation of the anthocyanin and flavonol biosynthesis pathway is presented in Fig 2

2.1.2.4 MOLECULAR CHARACTERIZATION AND EXPRESSION ANALYSIS OF KEY GENES OF ANTHOCYANIN BIOSYNTHESIS

The complex anthocyanin biosynthetic pathway is studied in detail and most of the biosynthetic genes have been elucidated by a number of investigators (Winkel – Shirley, 2001). The details regarding the key genes are presented below.

2.1.2.4.1 Chalcone synthase gene (*chs*)

The enzyme chalcone synthase, a plant specific polyketide, catalyzes the stepwise condensation of three acetate units starting from malonyl-CoA with p-coumaroyl-CoA to yield 4, 2', 4', 6'-tetrahydroxychalcone (Holton and Cornish, 1995).

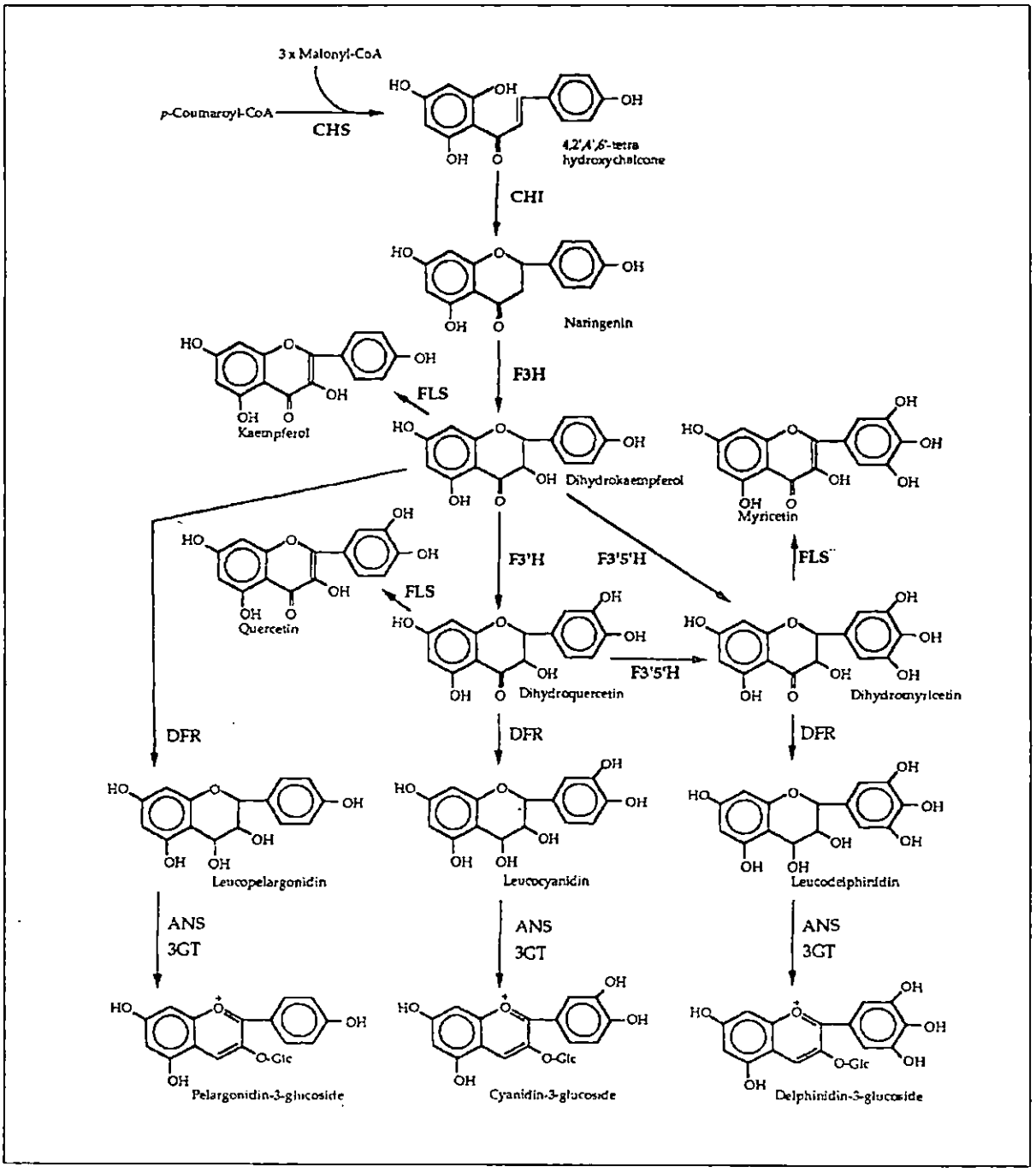

Figure 2. A diagrammatic representation of the anthocyanin and flavonol biosynthesis pathway (Holton and Cornish, 1995).

Kreuzaler et al. (1983) was successful in isolating a complete complementary DNA (cDNA) copy of *chs* messenger RNA (mRNA) from cultured parsley (*Petroselinum hortense*) cells. Using parsley cDNA as probe, Reif et al. (1985) isolated two *chs* gene from *Petunia hybrida*. The homologous cDNA fragments were cloned and the study showed that the two genes are not allelic, but members of a gene family. Cloning and characterization of *chs* gene in *Ipomea* were reported by Durbin et al. (1994). In *Ipomea* genome, the *chs* genes occur as a multigene family and the genes showed close relationship with petunia *chs-b* gene.

Reddy et al. (1996) reported the presence of CHS protein in rice seedling and its developmental stage- expression by western analysis. The CHS of rice was found to be immunologically similar to that of maize. Rosati et al. (1997)) isolated partial *chs* gene from petal tissues of *Forsythia intermedia* by Reverse Transcription – Polymerase Chain Reaction (RT-PCR). Northern blot analysis was carried out to study the expression pattern of the gene. They transformed colour mutant with these genes but the transformants did not accumulate any anthocyanin. They concluded that other genes or regulatory factors should be considered as responsible for the lack of anthocyanin production.

Wha-Shin Hsu et al. (1997) isolated and characterized a cDNA encoding *chs* homolog from *Phalaenopsis* orchid. Southern analysis revealed that a family of *chs* gene containing at least 10 complete members exists in the *Phalaenopsis* genome.

Lo et al. (2002) isolated and cloned a family of *chs* (*chs* 1-7) gene from *Sorghum bicolor*. The study suggested that all the seven genes occur as a cluster in the genome. *In silico* examination and expression analysis revealed that the genes were not differentially expressed, depending on the changes in growth and developmental conditions.

Han et al. (2006) successfully isolated a complete DNA sequence of *chs* (*pchs1*) from *Phalaenopsis* orchid. Nucleotide sequence analysis with other *Phalaenopsis* orchid cultivars revealed that *pchs1* (Gen Bank Accession No. AY82502) contained two exons and one intron at the conserved site. Southern blot analysis showed that at least four *chs* like genes are present in *Phalaenopsis* genome. By an RT-PCR study they found out that the *pchs1* gene is expressed in petals at early stages of flower development.

Chalcone synthase has been an attractive target for genetic engineering and there are numerous examples of co-suppression or down regulation of this gene, in order to modify flower colour towards pure white as a result of a complete absence of flavonoids (Tanaka et al., 2005).

2.1.2.4.2 Dihydroflavonol 4-reductase gene (*dfr*)

Dihydroflavonol 4-reductase (DFR) is one of the key enzymes involved in anthocyanin biosynthesis and proanthocyanin synthesis. DFR catalyzes the last common step in the flavonoid biosynthesis pathway leading to anthocyanins and proanthocyanidins (condensed tannins). In an NADPH-dependent reaction the enzyme reduces the three colourless dihydroflavonols - dihydrokaempferol (DHK), dihydroquercetin (DHQ), and dihydro-myricetin (DHM) to leucoanthocyanidins (Martin et al., 1985; Holton and Cornish, 1995 and Kristiansen and Rohde, 1991). These are subsequently converted to pelargonidin, cyanidin and delphinidin. In addition, they are the precursors for the production of catechins and proanthocyanidins, which are involved in plant resistance and are considered as potential health-protecting compounds in food and feed. *dfr* genes have been isolated from several higher plants (Helariutta et al., 1993; Sparvoli et al., 1994 and Rosati et al., 1997).

Helariutta et al. (1993) reported the cloning and characterization of *dfr* cDNA from the corollas of *Gerbera hybrida* Var Regina. Southern blot analysis showed that the plant harbour a small family of *dfr* genes and one of the members is mainly responsible for the *dfr* activity in corolla. The functionality of the clone was also tested *in-vivo* by complementing the dihydrokaempferol accumulating petunia mutant line RL01.

Characterization and expression analysis of cDNA encoding *dfr* were reported by Charrier et al. (1995) in *Medicago sativa*. Southern blot analysis indicated that the gene is represented by a single copy within the tetraploid genome. By a combination of Northern blot and RT-PCR analysis, it was revealed that the *dfr* genes are expressed in flowers, nodules, leaves and roots, with a pattern distinct from *chs* expression.

By an RT-PCR methodology Rosati et al. (1997) isolated a full length *dfr* from petals of *Forsythia intermedia*. The mRNA levels in petal tissues of wild and transgenic clones were quantitatively determined by competitive PCR assays with CaMV 35S promoter - driven *dfr* gene of *Antirrhinum majus*. The result showed that the gene shows its highest expression during early stages of anthesis.

Moyano et al. (1998) isolated a putative *dfr* gene from strawberry (*Fragaria* × *ananassa* cv Chandler) during fruit development. Northern analysis showed that, the gene is predominantly expressed in the biosynthesis of anthocyanin during early stages of fruit colour development. Zhuang et al. (1999) reported the isolation of full length cDNA sequence encoding a putative *dfr* gene from rice.

Himi et al. (2004) isolated a full length genomic sequence of three homologous *dfr* genes from grain tissues of hexaploid wheat. In wheat tissues these

three genes are located on different chromosomes and they show tissue dependent expression.

Mudalige-Jayawickrama et al. (2005) produced the first complete cDNA clone encoding *Dendrobium dfr*, isolated from floral buds of *Dendrobium*. The nucleic acid sequence of the cDNA showed 87 per cent similarity to *dfr* of *Cymbidium* and 84 per cent similarity to *Bromheadia* orchid. The expression of the gene by Northern blot analysis showed high activity throughout flower bud development but less in fully opened flowers and absent in leaves.

Ueyama et al. (2006) reported the cloning and characterization of cDNA encoding *dfr* gene from *Nierembergia* sp. Northern blot analysis revealed that the expressions of gene were coordinately regulated in parallel with the anthocyanin accumulation in the petals, indicating that anthocyanin biosynthesis is transcriptionally regulated.

Heiber et al. (2006) reported the expression of *dfr* gene on *oncidium* flowers. The study showed that the *dfr* gene *shows its* expression throughout the flower development and amplification of the full length DNA sequence revealed that three different genes were involved during flower colour development.

2.1.2.4.2.1 SUBSTRATE SPECIFICITY OF DIHYDROFLAVONOL 4-REDUCTASE (DFR)

Dihydro flavonol 4-reductase proteins of many plants can accept dihydroflavonols with different hydroxylation patterns namely dihydrokaempferol (DHK), dihydroquercetin (DHQ), and dihydromyricetin (DHM), as substrates. The three substrates of *dfr* are very similar in their structure; the difference is only in the

number of hydroxyl groups on the B phenyl ring, which is not the site of enzymatic action. Therefore, DFR from many species can utilize all the three substrates (Tanaka et al., 1998 and Johnson et al., 2001). However, *dfr* from some species such as *Petunia* and *Cymbidium* cannot reduce DHK efficiently, thus these species cannot produce pelargonidin based orange flower colour even if both *f3'h* and *f3'5'h* are absent (Forkmann and Ruhnau, 1987; Johnson et al., 1999 and Johnson et al., 2001).

Only a few pelargonidin accumulating flowers are found in *Dendrobium* (Kuehnle et al., 1997a). In *cymbidium* orchids the substrate specificity of *dfr* was investigated by transforming a mutant petunia line accumulating DHK as the major flavonol (Johnson et al., 1999). Thin layer chromatography (TLC) of transformed lines indicated that cymbidium *dfr* cannot efficiently reduce DHK and preferred DHQ as a substrate, resulting in the production of pink cyanidin instead of orange pelergonidin.

The diagram showing the chemical reaction catalyzed by dihydroflavonol 4-reductase gene is shown in Fig 3.

2.1.2.4.3 Flavonoid 3'5'- hydroxylase gene (*f3'5'h*)

Flavonoid 3', 5'-hydroxylase (*f3'5'h*), a member of the cytochrome p450 family, is the key enzyme in the synthesis of 3', 5'- hydroxylated anthocyanins, which are usually a prerequisite for the expression of blue or purple flowers (Su and Hsu, 2003).

Flavonoid 3', 5'-hydroxylase activity was first demonstrated with the microsomal fractions from flowers of *Verbena hybrida*, and further from the flowers

of *Callistephus chinensis*, *Lathyrus odoratus* and *Petunia hybrida* (Forkmann and Heller, 1999).

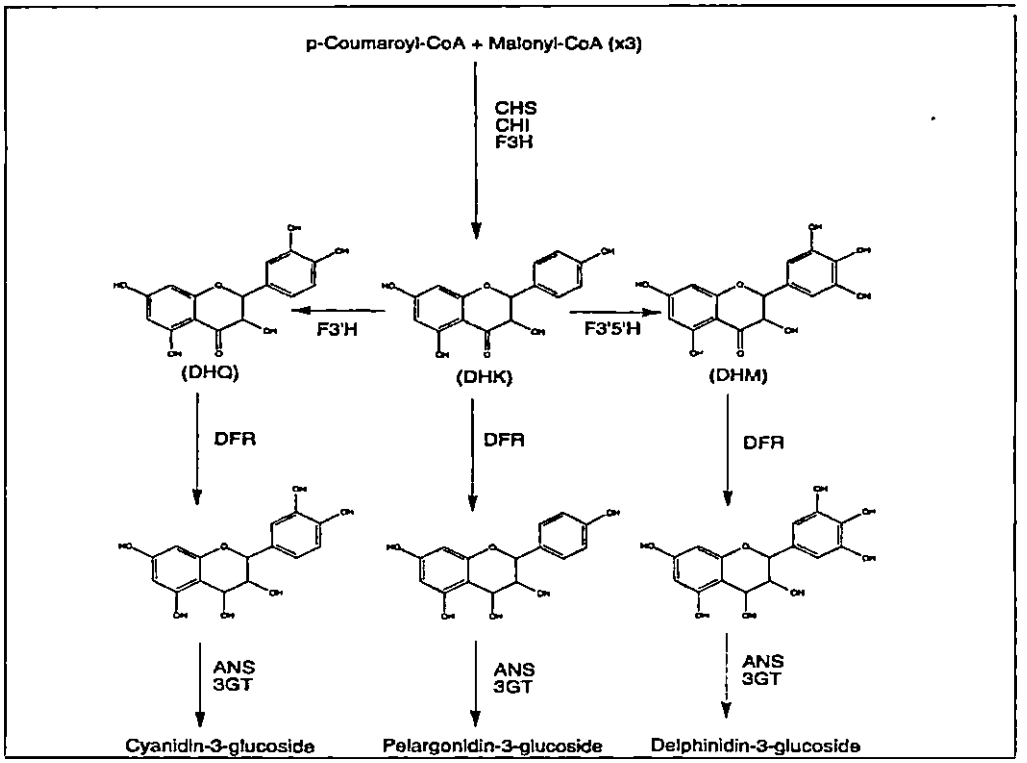

Figure 3. A schematic diagram showing the chemical reaction catalyzed by dihydroflavonol 4-reductase gene

Holton et al. (1993) isolated the petunia *f 3'5'h* (CYP75A1 and A3) for the first time. Petunia encode two *f 3'5'h* gene which are regulated by two loci, *hf1* and *hf2* and the *hf1* loci plays a dominant role. Tanaka et al. (1996) isolated a full length cDNA clone of *f3'5'h* gene from petals of *Gentiana triflora* and is enzymatically characterized by expressing cDNAs in a heterologous expression system.

Nielsen et al. (1997) isolated *f3'5'h* gene encoding cDNA sequences from petals of lisianthus (*Eustoma grandiflorum*). Northern blot analysis showed that the *f3'5'h* mRNA is transcribed late in the petal development.

Mori et al. (2004) successfully isolated and cloned a full length cDNA for *f3'5'h* gene from *Vinca major* by a PCR based strategy. Southern blot analysis showed that there are three to four copies of *f3'5'h* gene in the genome.

Complementary DNA clone encoding *f3'5'h* gene (designated as *ph35h*, Gen Bank Acc. No. DQ 148458 in) were isolated from *Phalaenopsis* orchids by Jingwen et al. (2006) by a PCR based strategy. Southern hybridization analysis indicated the presence of a single copy gene for *ph35h*. RT-PCR analysis showed that the *ph35h* mRNA is transcribed late during petal development. The transcript is abundant in the purple petals but not in leaves or root.

Many plant species such as rose, carnation, chrysanthemum, lack *f 3'5'h* activity and do not produce delphinidin, which indicates that these plants may have lost *f3'5'h* genes during their evolution (Tanaka, 2006).

2.1.2.5 FACTORS AFFECTING ANTHOCYANIN BIOCHEMISTRY AND EXPRESSION OF BIOSYNTHETIC GENES

The final colour which plants express is not only determined by the type and level of anthocyanins present in a particular species, but also by internal as well as external factors that modify their chromogenic properties (Robards and Antolovich, 1997).

2.1.2.5.1 INTERNAL FACTORS

2.1.2.5.1.1 pH

Vacuolar pH is a critical factor which regulates the flower colour. The vacuolar pH varies greatly among different species (from 4.3 to 7.8), and can also be different in different tissues or developmental stages (Tanaka et al., 1998). Vacuolar pH, that is most often maintained as weakly acidic, is critical to anthocyanin stability and colour. Although higher (neutral) pH generally yields bluer flower colours, anthocyanins are less stable at higher pH and must be stabilized with more than one glycosyl and aromatic acyl group (Goto and Kondo, 1991 and Honda and Saito, 2002). Increased pH moves the absorption spectra of cyanidin and delphinidin toward longer wavelength, making them appear darker (Mol et al., 1998).

Genetic control of petal vacuolar pH is known in petunia and morning glory. One of the key gene affecting the vacuolar pH have been cloned from Japanese morning glory (*Ipomea nil*) by transposon tagging (Yamaguchi et al., 2001). The *Ipomea* floral epidermal cells shift from reddish purple buds to blue open flowers with an increase in the vacuolar pH from 6.6 to 7.7 (Asen et al., 1979 and Yoshida et al., 1995).

In petunia, 7 loci (ph1-ph7) affecting the vacuolar pH have been identified (Mol et al., 1998). Recessive mutations in these pH loci display blueing of flower colours due to increased pH in the vacuole (Houwelingen et al., 1998).

2.1.2.5.1.2 Co-pigments and metal ions

Anthocyanins form complexes with co-pigments such as flavones and flavonols by aggregation, resulting in shift of the visible absorption maximum of the complex towards longer wavelength (Yu et al., 2006). This usually lead to darker flower colours (Forkmann, 1991). Flavones are common co-pigments that form complexes with anthocyanins.

Many metal ions including copper, calcium, aluminium, iron, magnesium and molybdenum were found to co-exist with anthocyanins (Ellestad, 2006). Such associations have a significant impact on flower colour. Sachray et al. (2002) studied the combined effect of elevated temperature and increased metal ion concentration on the accumulation of anthocyanins in aster flowers. They found that magnesium treatment of aster plants or detached flower buds partially prevents colour fading at elevated temperatures.

The effect of selenium on anthocyanin and chlorophyll content in maize was reported by Nowak (2008). He found that the increase in selenium concentration causes a dramatic increase in anthocyanin content and decrease in chlorophyll content. The experimental evidences proposed that this effect could be used as a test parameter to detect selenium toxicity in maize plants.

2.1.2.5.1.3 Super molecular structure

Under suitable pH, the vacuolar anthocyanins, co-pigments, and metal ions can assemble into stable, non-covalent super molecular complexes with distinct optical characteristics. Several of these structures have been isolated, crystallized, and structurally resolved (Ellestad, 2006). Structural differences that affect the

colour of anthocyanins are methylation and hydroxylation. Methylation produced less blueness, whereas, increased hydroxylation results in a marked increase in blueness (Yu et al., 2006)

2.1.2.5.2 ENVIRONMENTAL FACTORS

Expression of the flavonoid biosynthetic pathway is precisely regulated, in response to environmental factors (Robards and Antolovich, 1997). The environmental factors include the following.

2.1.2.5.2.1 Light

Studies have shown that light induces anthocyanin accumulation in green tissues and cultured cells *via* the activation of anthocyanin biosynthetic genes (Mol et al., 1996). The effect of light irradiation on anthocyanin production in suspended cultures of *Perilla frutescens* was done by Zhong et al. (1991). The study showed that the cultures accumulate more anthocyanin on illumination.

Biran and Halevy (1974) showed that covering rose flowers does not inhibit petal growth and pigmentation, where as covering or removing leaves decreases flower fresh weight and anthocyanin content. Effect of light on *Arabidopsis* seedlings was studied by Kubasek et al. (1998). The study showed that, flavonoid genes are transiently expressed during germination in a light-dependent manner, with maximum mRNA levels occurring in 3-day-old seedlings.

2.1.2.5.2.1.1 Light quality

Experiment conducted by Batschauer et al. (1991) to understand the light dependent expression of chalcone synthase in mustard revealed that phytochrome controls expression of this gene in cotyledons of mustard seedlings. However, blue/UV- light photoreceptors control expression in later stages of development. White and red light induce anthocyanin accumulation and anthocyanin biosynthetic gene transcription in egg plant (*Solanum melongena*) hypocotyls (Toguri et al., 1993) and tomato seedlings (Bowler et al., 1994). Blue and red light had similar effect on transcript accumulation; whereas the effect of green light was slightly lower in detached petunia petals (Moscovici et al., 1996).

2.1.2.5.2.1.2 Light intensity

Light and sucrose when applied in combination induced the *dfr* gene in juvenile phase lamina tissue of English ivy (*Hedera helix*), but sucrose did not induce the gene in the dark (Murray and Hackett, 1991). Gong et al. (1997) reported the effect of light on the expression of the anthocyanin biosynthesis genes. He could observe that under weak light conditions, the accumulation of both anthocyanin and mRNA of all biosynthetic enzymes was lower in leaves of the red form of *Perilla frutescens*.

2.1.2.5.2.2 Temperature

Temperature plays a key role in anthocyanin accumulation in purple rice grains (Phoka et al., 2002). RT-PCR analysis revealed suppression of *dfr* expression under high temperature and desuppression in cool season. Tissues collected from plants grown under high temperature were deep purple in colour.

Yamane et al. (2006) studied the effect of temperature on accumulation of anthocyanins in grape berry skins. The study showed that, at lower temperatures the fruit skin accumulates more anthocyanin compared to higher temperature.

2.1.2.5.2.3 Sugars

The interrelationships between developmental, environmental, and metabolic signal transduction pathways control the production of flavonoids. Sugars act as signaling molecules, whose signal transduction pathways may lead to the activation or inactivation of gene expression (Solfanelli et al., 2006).

Increased sucrose concentration enhanced petal growth and pigmentation in detached flowers of *Rosa hybrida* (Kuiper et al., 1991), *Liatris spicata* (Han, 1992), *Eustoma grandiflorum* (Kawabata et al., 1995) and *Hyacinthus orientalis* (Hosokawa et al., 1996). Several studies indicate that sugar level increases during petal development to a maximum at the stage of rapid cell expansion (Tsukaya et al., 1991 and Clement and Mabry, 1996). Weiss (2000) reported the effect of sugars on accumulation of anthocyanins in petunia flowers. The study showed that in addition to acting as transcription inducer for the accumulation of anthocyanin in flowers, sugars also induces cell expansion.

Genes coding for *dfr* and *ans*, were up-regulated and the accumulation of anthocyanins was strongly increased by sucrose in grape (*Vitis vinifera*) cells (Gollop et al., 2002), and signal transducers, such as Ca^{2+} and protein kinases/phosphatases, were also involved in this process. Whole-genome transcript profiling studies in *Arabidopsis* revealed that the flavonoid and anthocyanin biosynthetic pathways are

strongly up-regulated following sucrose treatment. Besides mRNA accumulation, sucrose affects both flavonoid and anthocyanin contents (Solfanelli et al., 2006).

2.2 METABOLIC ENGINEERING FOR FLOWER COLOUR

Modification of anthocyanin biosynthetic pathway is the most reported type of genetic modification in plants. Most of the genes encoding different enzymes of this pathway are characterized and the increasing knowledge about these genes is widely used to engineer newer traits to increase the consumer acceptability. The different approaches include introduction of biosynthetic genes from other species, sense or antisense suppression of biosynthetic genes, down regulation of genes by RNA interference (RNAi) and transposon mediated flower colour engineering (Tanaka et al., 2005).

2.2.1 Introduction of biosynthetic genes from other species

The first report on flower colour modification using genetic engineering was done in petunia where *dfr* gene (*al*) from *Zea mays* was introduced into a petunia mutant that accumulates dihydrokaempferol (DHK) to produce brick-red coloured flowers (Meyer et al., 1987). It was used to overcome the substrate specificity of petunia dihydro flavonol 4-reductase (*dfr*), allowing the flowers to accumulate orange pelargonidin based anthocyanins in a mutant that lack flavonoid hydroxylase activity. Later, traditional breeding of these transgenic plants provided attractive orange colored petunia flowers (Griesbach and Klein, 1993 and Johan et al., 1995). Introduction of rose and gerbera *dfr* gave a more consistent and stronger expression in petunia (Elomaa and Holton, 1995).

Holton et al. (1993) reported the expression of a petunia *f3'5'h* gene in a pale pink petunia line deficient in *f3'5'h* and *f3'h*, elevated the amount of delphinidin – based anthocyanins and yielded reddish purple flowers. Pink to magenta colour changes in petunia were achieved using petunia and lisianthus *f 3'5'h* genes (Shimada et al., 1999). Expression of a *Vinca major f3'5'h* gene in red – flowered petunia yielded deep red flowers with deep purple sectors (Mori et al., 2004).

Some major ornamental species such as rose, chrysanthemum, and carnation have little or no delphinidin in their flowers and lack blue flowers. Successful introduction of the *f3'5'h* gene that produces delphinidin has been accomplished in carnation by Florigene Ltd., Australia and Suntory Ltd., Japan (Tanaka et al., 2005).

Introduction of *Petunia f3'5'h* and *dfr* genes in to a *dfr* deficient white carnation has enabled the production of mauve coloured carnation flowers (Tanaka et al., 1998). Commercial varieties named Moondust and Moonshadow are marketed in Australia, Japan and USA (Tanaka et al., 1998). However, introduction of the *f3'5'h* gene alone might be insufficient to produce a true blue flower in ornamentals. Introduction of petunia *f3'5'h* together with petunia cytochrome b_5 increased the activity of the introduced *f3'5'h* in transgenic carnation (Brugliera et al., 2000). Other factors such as vacuolar pH, co - pigmentation, and intermolecular factors are also important in getting a true blue color (Brouillard and Dangles, 1994).

Transformation of a pink *Lobelia erinus* with a lisianthus *f3'5'h* gene under the control of a CaMV35S promoter produced blue coloured flowers (Kanno et al., 2003).

Genetic engineering of coloured flavonoids such as chalcones and aurones resulted in yellow coloured flowers in many of the ornamentals. Chalcones are

unstable intermediates involved in flavonoid biosynthesis and appear colourless or light yellow. Several important ornamentals such as pelargonium, cyclamen, lisianthus and impatiens do not have yellow coloured flowers. Transgenic plants that accumulate yellow coloured flavonoids were produced by introducing a *chs* cDNA from *Medicago sativa* in to a white flowered petunia mutant (Davies et al., 1998). Transgenic plants accumulated a new flavonoid 6'-deoxychalcones, instead of more common 6-hydroxychalcone. Deoxychalcone is not accepted as a substrate by chalcone isomerase, there by reducing the flow of intermediates towards anthocyanins and allowing light yellow chalcones to accumulate in flowers.

The invariably yellow colouration of *Forsythia* petals is caused by accumulation of carotenoids, and lack of anthocyanin. However some anthocyanins are formed in the sepals. Studies on this flavonoid pathway revealed that anthocyanin formation in the petals is impaired at the *dfr* and, in particular, the *ans* steps. Introducing the *dfr* gene from *Antirrhinum majus* and the *ans* gene from *Mathiola incana* resulted in transgenic *Forsythia* with slightly brownish flowers (Rosati et al., 2003).

2.2.2 Sense or antisense suppression of biosynthetic genes

Expression of a gene can be inhibited by introducing a gene of interest or a close homologue of it, in sense or in antisense direction (Mol et al., 1990). Efficiency of the suppression was shown to be dependent on the transgene promoter strength and homology between the transgene and the native gene (Que et al., 1997).

Inhibition of pigment synthesis by introduction of sense and antisense *chs* or *dfr* was achieved in *Petunia hybrida* (Van Blokland et al., 1994), *Dianthus caryophyllus*, *Eustoma grandiflorum*, *Gerbera hybrida*, *Torenia hybrida* and *Rosa*

hybrida (Davies and Schwinn, 1997; Elomaa and Holton, 1995 and Tanaka et al., 1998). White flowers were obtained in *Dianthus caryophyllus*, while white patterned phenotypes resulted in *Petunia hybrida, Eustoma grandiflorum* and *Torenia hybrida*. Some transformants of *Gerbera hybrida, Rosa hybrida, Dianthus caryophyllus* and *Torenia hybrida* gave pale coloured phenotypes due to the reduction of anthocyanin synthesis throughout the flower development (Tanaka et al., 1998).

Introduction of flavonol synthase *(fls)* gene in antisense direction has resulted in higher anthocyanin content in petunia (Davies et al., 2003), tobacco (Holton et al., 1993) and lisianthus (Nielsen et al., 2001). A similar result was reported by sense suppression of flowers in *Petunia cv. Surfinia* Pink where flower colour changed from pink to red purple (Tanaka et al., 1998). Nishihara et al. (2006) produced transgenic white flowered gentian plants by the antisense suppression of *chs* gene.

2.2.3 Down regulation of genes by RNA interference (RNAi)

RNA interference has been developed as a powerful tool for down regulation of a target sequence (Wang and Water house, 2001) in plants. In some cases the frequency of phenotypic changes is typically more than 50 per cent and the phenotype is more stable than that obtained using antisense/co-suppression (Mizutani et al., 2003). RNAi has been applied to suppress some structural genes in anthocyanin biosynthesis, causing the inhibition of anthocyanin accumulation and a change in flower colour in transgenic plants (Fukusaki et al., 2004; Tsuda et al., 2004; Nishihara et al., 2005 and Nakamura et al., 2006).

Down regulation of *f3'5'h* and *f3'h* gene using RNAi suppression resulted pale pink flowers in torenia due to the accumulation of pelargonidin based

anthocyanins. By the addition of *dfr* gene from rose or pelargonium resulted a brighter colour (Ueyama et al., 2002).

Nakamura et al., (2006) reported that RNAi suppression of *ans* gene in *Torenia hybrid* yielded white flower colour. Miki et al. (2005) have developed novel RNAi constructs, which could induce simultaneous silencing of up to four genes, using chimeric triggers of *OsRac* genes in rice. Using this silencing method, Nakatsuka et al. (2007) successfully produced red flowered tobacco plants using a chimeric RNAi construct for the suppression of two genes (*f 3'h* and *fls*) and expression of a gerbera *dfr* gene. The transgenic plants produced high amount of additional pelargonidin and was confirmed by High Performance Liquid Chromatography (HPLC) analysis.

2.2.4 Transposon mediated flower colour engineering

Variegated phenotypes in flowers and leaves are highly valued in ornamental plants. Insertion of a transposon into a flavonoid biosynthetic gene or a regulatory gene of the biosynthetic pathway in morning glory resulted in flowers with white sectors in a coloured background (Iida et al., 1999).

Transforming tobacco using a binary vector containing the *Arabidopsis* transposon *Tag 1* that was inserted between a CaMV35S promoter and the maize *R* gene resulted in variegated flower colour (Liu et al., 2001). In the resulting transgenic petunias, the *R* gene, a dominant positive regulator of anthocyanin biosynthetic genes is transcribed only when *Tag 1* is excised. Half of the transgenic plants exhibited variegated flower pattern. Each line had a different pattern, with varying intensities of colour.

2.3 METABOLIC ENGINEERING IN ORCHIDS

Orchid floriculture industry is highly priced and new varieties have very high demand in the international market. Development of new varieties through metabolic engineering is gaining importance. Kuehnle and Sugii (1992) were the first to report the possibility of applying genetic transformation in *Dendrobium* orchid for potential trait improvement using particle bombardment. Subsequently, other orchid generas like *Phalaenopsis* (Anzai et al., 1996 and Liao et al., 2004), and *Cymbidium* (Yang et al., 1999), were also transformed by particle bombardment using mainly GUS as the reporter system.

Chia et al. (1994) reported the genetic transformation in orchid using firefly luciferase gene. When these plants are sprayed with the substrate luciferin, the plants emit a soft light (grown in the dark). *Agrobacterium* mediated genetic transformation have been studied in *Dendrobium* x Madame Thong –In (Yu et al., 2001), *Oncidium* (Liau et al., 2003a, b) and Phalaenopsis (Chan et al., 2005). Semiarti et al. (2007) reported the *Agrobacterium* mediated genetic transformation of wild species of Phalaenopsis (*P. amabilis*) using PLBs.

Seed imbibition, electro injection and pollen tube mediated transformation are the alternative method for transformation, but the low recovery rate of a putative transformant makes this method ineffective in *Dendrobium* orchids (Nan and Kuehnle, 1995). The study in *Phalaenopsis* by using pollen tube mediated transformation indicated that this method is a useful transformation method for producing transgenic *Phalaenopsis* plants (Hsieh and Huang, 1995). In *Calanthe*, electrophoresis of protocorms resulted in a transformation frequency of about 50 per cent, vs. 3.3 per cent by particle bombardment (Griesbach, 1994).

2.4 GENE ISOLATION AND CHARACTERIZATION

Isolation of specific genes and its sequence knowledge is an important step towards molecular manipulation studies. Several techniques are developed for the isolation and characterization of genes. Among these, RT-PCR is a reliable technique, which makes use of reverse transcriptase for the synthesis of cDNA from mRNA transcripts (Avila and Canovas, 2000).

2.4.1 RT-PCR (Reverse Transcription –Polymerase Chain Reaction)

Reverse Transcription –Polymerase Chain Reaction is a sensitive technique in molecular biology for the detection and quantitation of specific mRNA. RT-PCR is highly specific and is the frequently used method for gene expression studies (O'Driscoll et al., 1993). Compared to Northern blot analysis and RNase protection assay, RT-PCR can be used to quantify mRNA levels from much smaller samples (Avila and Canovas, 2000). The first step in any RT-PCR reaction is the enzymatic conversion of RNA to single stranded DNA. Oligo d(T), which binds to the endogenous poly $(A)^+$ tails of eukaryotic mRNAs can be used as a universal primer for first strand synthesis (Bank et al., 1972). The particular cDNA can be further amplified using specific primers (Sambrook and Russel, 2001).

Enzymes coded by Avian myeloblastosis virus (AMV) , and the Meloney strain of murine leukemia virus (M-MuLV), enzymes are commonly used for reverse transcription reaction to synthesize cDNA and then a second reaction is performed using one of the thermostable DNA polymerases. Kotewicz et al. (1988) reported that the enzyme coded by AMV has a powerful RNaseH and endonuclease activity that can cleave the RNA-DNA hybrids and restrict the length of cDNA. Gerard et al. (1997) found that the murine enzyme was better suited for RT-PCR because it lacked

RNase H activity. However, the murine enzyme reaches maximum activity at lower temperature than the AMV reverse transcriptase and hence is not suited for RNA template with secondary structure.

2.4.1.1 Primer designing

Primer designing is the single largest variable in PCR application and is the most important factor in determining the result of PCR reactions. Gene specific primers are designed based on the conserved sequence present among the gene of interest (Sibhatu, 2003). Gen Bank (http://www.ncbi.nlm.nih.gov) is one of the largest data banks of DNA sequences hosted by National Centre for Biotechnology Information (NCBI). It provides the facility for the comparison of DNA sequences of related or distantly related species (Rastogi, 2004). Multiple sequence alignment programs can be used to locate the sequence pattern conserved through evolution (Higgins et al., 1988). Clustal series of programmes are widely used in molecular biology for the multiple alignments of both nucleic acid and protein sequences (Chenna et al., 2003).

Among the different software used for designing primers for PCR reactions, Primer 3 is commonly used software (http://fokker.wi.mit.edu/primer3/) which designs primers according to the conditions specified by the user. Primer3 considers conditions like melting temperature, concentration of various solutions in PCR reaction, primer bending and folding, and many other conditions when attempting to choose the optimal pair of primers for a reaction. The software was originally developed by Rozen and Skaletsky (2000) at the Whitehead Institute for Biomedical Research, USA.

Innis et al. (1990) suggested a set of rules for designing primers which include the following: primer length should be 17-28 bases, base composition should be 50-60 per cent (G+C), primers should end (3') in a G or C, or CG or GC, Tms between 55-80° C are preferred, 3'-ends of primers should not be complementary, primer self-complementarity should be avoided, runs of three or more Cs or Gs at the 3'-ends of primers should be avoided.

2.4.2 RNA EXTRACTION

Recovering high quality, intact RNA free from genomic DNA, protein and secondary metabolite contamination is the most critical step in performing RT-PCR (Salzman et al., 1999)

2.4.2.1 Problems encountered during RNA extraction

Isolation of good quality RNA from plant tissues is problematic due to their hard cell wall, which is very difficult to disrupt. Plant cells often contain significant amounts of compounds such as tannins, phenolics, and complex polysaccharides that can affect RNA quality and inhibit downstream reactions. Phenolic compounds are readily oxidized and covalently linked with quinines, and to nucleic acids, thereby interfering RNA retrieval (Loomis, 1974 and Salzman et al., 1999).

To obtain good preparations of eukaryotic messenger RNA (mRNA) it is necessary to minimize the activity of ribonucleases (RNases) liberated during cell lysis by using inhibitors of RNases or methods that disrupt cells and inactivate RNases simultaneously (Sambrook and Russel, 2001). Ribonuclease A can be temporarily denatured under extreme conditions, but it readily renatures. RNase A

can survive autoclaving and other standard methods of protein inactivation (Farrel, 1998).

General laboratory glassware and plastic ware are often contaminated with RNases and hence should be treated by baking at 180° C for eight hours (glassware) or by rinsing with chloroform (plastic ware). Fedorcsak and Ehrenberg (1966) identified diethyl pyrocarbonate (DEPC) at the concentration of 0.1 per cent in water as a strong inhibitor of RNases. Diethyl pyrocarbonate destroys enzymatic activity by modifying -NH, -SH and -OH groups in RNases and other proteins. After treatment, the DEPC filled glassware or plastic ware should be allowed to stand for two hours at 37° C followed by incubation at 100° C for 15 minutes or autoclaved till DEPC scent was removed (Kumar and Linderberg, 1972). These treatments remove traces of DEPC that might otherwise modify purine residues in RNA by carboxymethylation.

Processing of frozen tissue samples in RNA lysis buffer containing guanidium, lithium or SDS could minimize the problem of RNases (Sambrook and Russel, 2001). Proteins dissolve readily in solutions of potent denaturing agents such as guanidium hydrochloride and guanidium thiocyanate (Cox, 1968). RNaes are inactivated by 4M guanidium thiocyanate and reducing agents such as B-mercaptoethanol (Sela et al., 1957). This combination can be used to isolate intact RNA from tissues rich in RNases (Chirgwin et al., 1979).

2.4.2.2 Protocols for the extraction of plant RNA

Numerous standard protocols have been developed for the effective isolation of high quality RNA suitable for functional genomics based experiments (Chomczynski and Sacchi, 1987; Logemann et al., 1987; Ainsworth, 1994 and

Sambrook and Russel, 2001). Efficiency of these methods varies with the types of tissue. Most published protocols, for RNA isolation, have used strong protein denaturants and guanidine/guanidium salt for RNase inactivation.

Presence of large quantities of polysaccharides and polyphenolic compounds that accumulate and co-precipitate during RNA extraction will affect both quantity and quality of RNA isolated (Asif et al., 2000 and Logemann et al., 1987).

Wang and Vodkin (1994) demonstrated that high quality RNA could be extracted from soybean by inhibiting polyphenolic compounds in the presence of bovine serum albumin and polyvinyl pyrrolidone (PVP) in conjunction with phenol extraction and overnight Lithium Chloride (LiCl) precipitation. Ainsworth (1994) suggested that the extraction of RNA at high temperatures can reduce the interference of polysaccharides and polyphenols to a greater extent. He succeeded in isolating high quality RNA from the sorrel (*Rumex acetosa*) floral tissue rich in polysaccharides using a method in which RNA extraction was carried out at 65° C to prevent polysaccharide precipitation.

Gehring et al., (2000) described the use of high molecular weight polyethylene glycol (HMW-PEG) to isolate RNA from plant tissues like *Aloe vera, Anona, Clusia, Euphorbia* etc. Method developed by Schneitz (2000) utilizes hot phenol maintained at 80° C for isolation of total RNA from plant tissues rich in polysaccharides. Using a Cetyl Trimethyl Ammonium Bromide (CTAB) based extraction protocol with 3M Sodium acetate Asif et al. (2000) isolated good preparations of RNA from banana fruit tissues.

Malnoy et al. (2001) isolated RNA from pear leaves using phenol and PVP to remove proteins and polyphenols. The isolated RNA was further purified by LiCl,

with a 2-butoxyethanol treatment between the LiCl steps. Iandolino et al. (2004) developed a CTAB based method for isolating RNA from Grape vine tissues (*Vitis vinifera* L.) The extraction buffer containing CTAB and beta-mercaptoethanol are pre-heated to 65° C and precipitation with LiCl.

Meisel et al. (2005) compared the efficiency of three different protocols for RNA isolation in peach (*Prunus persica*). The first two methods, phenol-chloroform extraction and LiCl precipitation protocol (Das et al., 1990) and guanidium thiocyanate/phenol–chloroform extraction protocol (Chomczynski and Sacchi, 1987), resulted in total RNA that was contaminated with large quantities of polysaccharides and polyphenolic compounds. The third protocol which was a combination of several other RNA isolation protocols (Chang et al., 1993; Salzman et al., 1999 and Zeng and Yang, 2002) yielded high quality total RNA. The extraction was carried out using CTAB buffer pre-heated to 65° C, followed by addition of PVP and ethanol precipitation.

Wang et al. (2005) developed a method to isolate RNA in good yield and integrity from *Ginkgo biloba* leaves containing high levels of flavonoid glycosides, terpenes, lactones, carbohydrates and polyphenolics as secondary metabolites. Polyvinyl pyrrolidone at 2 per cent and beta-mercaptoethanol at 4 per cent were added to the standard CTAB extraction buffer. Ethanol/acetate was used to precipitate RNA and using a phenol- chloroform extraction the co-precipitated polysaccharides were removed.

Heiber et al. (2006) describe the use of TRIZOL reagent for the extraction of good quality intact RNA from flower buds of *Oncidium* orchid. Using the same reagent Han et al. (2006) also isolated good preparations of RNA from *Phalaenopsis*. orchid.

2.4.2.3 RNA storage

Proper storage of RNA samples can minimize the problems of degradation. Isolated RNA can be dissolved in RNase free water (with 0.1 mM EDTA) or TE buffer (10Mm Tris, 1mM EDTA) and stored at -20° C for a shorter period of time (Farrel, 1998). Storage buffer containing divalent cations such as Mg^{+2} and Ca^{+2} will prevent heat-induced strand scission (Sambrook and Russel, 2001). RNA is stable at -80° C for up to one year (Farrel, 1998). Chomczynski, (1992) reported that the solubilization in formamide protect RNA from degradation. Storage of RNA at -20° C as ethanol precipitate can improve the storage for longer periods of time.

2.4.3 MESSENGER RNA (mRNA) PURIFICATION

In contrast to ribosomal RNA (rRNA) and transfer RNA (tRNA), mRNA carries a tract of poly(A)$^+$ at their 3' termini. mRNA's can therefore be separated from the bulk of cellular RNA by affinity chromatography on oligo d(T) cellulose (Edmonds et al., 1971 and Aviv and Leder, 1972). This poly(A)$^+$ tail is essential for the preparation of cDNA. Poly(A)$^+$ RNA gives better results in reverse transcription, compared to total RNA.

Many alternative methods to oligo d(T) chromatography have been devised over the years to select poly(A)$^+$ mRNA from preparations of total RNA. Linderberg and Pesson, (1974) developed a technique in which instead of oligo d(T) cellulose, poly(U) - sepharose was used to bind poly(A)$^+$ mRNA. Wreschner and Herzberg (1984) developed a method to elute poly (A)$^+$ mRNA from small quantities of total RNA. They spotted total RNA on to a paper filter to which poly (U) residues were covalently attached. The filters were then washed with DEPC treated 0.1 M NaCl and 70 per cent ethanol. Poly(A)$^+$ RNA was then eluted by heating the filters at 70°C for 5 minutes in water.

Elution with streptavidin coated paramagnetic beads is another commonly used poly(A)$^+$ mRNA purification method (Albertsen et al., 1990; Hornes and Korsnes, 1990 and Jacobsen et al., 1990). Using this method poly(A)$^+$ mRNA can be isolated directly from the lysis buffer. Biotinylated oligo d(T) primer is added directly to the lysate and allowed to hybridize with the poly(A)$^+$ tails of cellular mRNA. Magnetized beads to which streptavidin has been coupled are added to the lysate. The streptavidin captures the biotynylated oligo d(T) – poly(A)$^+$ mRNA complexes and affixes them to the magnetized beads. A magnet is then used to retrieve the beads from the lysate solution and washed with high salt solution. In the final step the poly(A)$^+$ mRNA is released from the beads with water and then collected by ethanol precipitation.

2.4.4 CLONING OF PCR PRODUCT

The cloning of a DNA fragment in to a plasmid vector is a routine procedure in recombinant DNA technology. Cloning of amplified segment of DNA generated by PCR in to vector can be achieved by several methods.

Hemsley et al. (1989) described a method for cloning PCR products in which the 3' -5' exonuclease activity of bacteriophage T4 DNA polymerase was used to remove extended bases present in the PCR product. The polished DNAs could then be phosphorylated by T4 polynucleotide kinase and cloned in to a blunt ended dephosphorylated vector. Hinnisdaeles et al. (1996) used *Pwo* and *Pfu* Taq DNA polymerase for cloning blunt ended DNA fragment. The cloning efficiency was found to be 10-100 folds lower than the efficiencies attained with DNAs equipped with cohesive termini.

Another method for cloning PCR products in to vector is by adding restriction sites to the 5' termini of the oligonucleotides used to prime PCR. The method was originally developed by Scharf et al. (1986). The primer specific restriction site was transferred to the termini of the target DNA during amplification. These fragments were then cleaved with appropriate restriction enzymes to generate amplified fragments of DNA with cohesive termini because the restriction site may identical in two primers, thus no difference allows the ligation of the termini of the amplified DNA fragment to the vector.

The most common method for cloning requires the use of DNA ligase to covalently link the compatible ends of the DNA fragment and the linearized plasmid, forming a single cyclic molecule that is capable of autonomous replication in host cells (Ausubel et al., 1987). Holton and Graham (1991), Marchuk et al. (1991), and Mead et al. (1991) observed that many DNA polymerases, including *Thermus aquaticus* (Taq) DNA polymerase, are capable of adding an additional non-template directed nucleotide to the 3' ends of a blunt ended DNA fragment. Clark (1988) and Hu (1993) identified this additional nucleotide as adenosine. Most of the PCR products amplified by Taq DNA polymerase thus possess a single 3'- A overhang at both ends. To directly clone PCR products which have 3'- A overhangs, a linearized T-vector which has a 3'-T overhang at each end can be used. The complementarity between the vector 3'–T overhangs and the PCR product 3' A overhangs allows direct ligation of Taq amplified PCR products in to the vector, and this strategy is commonly referred to as 'TA cloning (Holton and Graham, 1991; Marchuk et al., 1991 and Mead et al., 1991).

In addition to the basic methods a large variety of techniques to clone amplified fragments have been published, which include ligation independent cloning (Aslanidis and de Jong, 1990; Haun et al., 1992; Kaluz and Flint, 1994 and

Schuldiner and Tanner, 1997), UDG cloning (Nisson et al., 1991 and Smith et al., 1993), directional cloning using exonuclease III (Kaluz et al., 1992), *in vivo* cloning (Jones and Howard, 1991) and turbo cloning (Boyd, 1993).

2.4.5 SEQUENCING

DNA sequencing is a biochemical method for determining the order of the nucleotide bases, adenine, guanine, cytosine, and thymine, in a DNA oligonucleotide. The two rapid sequencing techniques in current use are the enzymatic method of Sanger et al. (1977) and the chemical degradation method of Maxam and Gilbert in (1977). Both these methods generate populations of radiolabeled oligonucleotides having variable terminus and are resolved by electrophoresis. Modification to Sangers method of sequencing was the use of fluorescent labeled dye terminators instead of radio labeling (Smith et al., 1986 and Prober et al., 1987). In dye-terminator sequencing, each of the four dideoxynucleotide chain terminators is labeled with fluorescent dyes, each of which with different wavelengths of fluorescence and emission. In automated DNA sequencing the sequencer will detect and record the dye fluorescence and data output as fluorescent peak trace chromatograms.

Materials and methods

3. MATERIALS AND METHODS

The study entitled "Isolation and characterization of cDNA encoding dihydroflavonol 4-reductase gene from orchid *Dendrobium* variety Sonia 17" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram, during the year 2006 to 2008. Details regarding the experimental materials used and the methodology adopted for various experiments are presented in this chapter.

3.1 MOLECULAR ANALYSIS

3.1.1 Designing and synthesis of gene specific primer

Primers for dihydroflavonol 4-reductase were designed using Primer 3 software (Rozen and Skaletsky, 2000). Forward and reverse primers were designed based on the conserved regions of orthologous gene sequences collected from the Gen Bank. Heterologous cDNA sequences from various plant species were downloaded from the National Centre for Biotechnology Information (NCBI) Gen Bank in FASTA format. Homologous regions of the genes were identified using Clustal X 1.81 (Thompson et al., 1997) multiple sequence alignment program. The conserved regions were selected for designing primers with Primer 3 software. BLASTN analysis was also carried out to find the binding specificity of the designed primer. Oligonucleotide properties calculation programme (www.basic.northwestern.edu/biotools/oligocalc.html) was used to find the 3' complementarity, hairpin formation and self annealing of the primer pairs. Three primers were also designed using primer3 software.

The following criteria were specified for designing *dfr* primer sets

1. Primer size: 20-22 bases
2. Product size: 500-800 bases

3. Primer GC per cent: 50-60 per cent
4. Maximum allowable length of a mononucleotide repeat: 4
5. Number of consecutive Gs and Cs at the 3' end of both left and right primer: 1

Other parameters were set as default. The primers designed were synthesized and supplied by Integrated DNA technologies (IDT), USA.

3.1.2 REVERSE TRANSCRIPTION -PCR

3.1.2.1 MATERIALS

3.1.2.1.1 Plant material

The *Dendrobium* Sonia 17, orchids were purchased from an authenticated orchid grower and were maintained at the green house of College of Agriculture, Vellayani, Thiruvananthapuram. Inflorescence was used for isolating RNA. All the stages of flower buds were used for RNA isolation. Either fresh flower buds or those stored at -80° C were used. Flower buds were washed with sterile DEPC - treated water before the isolation steps.

3.1.2.1.2 Equipments

1. Programmable thermal controller (PTC 100, MJ Research)
2. Spectrophotometer (Spectronic, Genesys 5)
3. Centrifuge (Eltek RC 4100D)
4. Circulating water bath (Cyberlab)
5. Gel documentation system (Bio-RAD)
6. Electronic balance (Shimadzu Corporation)
7. Deep freezer -20° C (Rotek) and -80° C (Sanyo ultra low)
8. Laminar Air Flow Chamber (TPL, Thermadyne)

3.1.2.1.3 Chemicals

1. mRNA purification kit (KT 80, Genei, Bangalore)
2. RT-PCR Kit (KT 74, Genei, Bangalore)
3. pGEM-T Easy Vector cloning kit (Promega corporation)
4. 100 bp DNA molecular size marker (Genei, Bangalore)
4. TRIZOL reagent (Invitrogen)
5. Diethyl pyrocarbonate (DEPC) (Sisco Research Laboratories)
6. Tris-HCL, EDTA and SDS (Sisco Research Laboratories)

3.2.2.2 RNA ISOLATION

Isolation of good quality intact RNA free of protein, genomic DNA and secondary metabolite contamination is the most critical step in gene isolation. To isolate high quality RNA, it is essential that RNases are not introduced into RNA preparations. RNases are very stable enzymes that catalyze the degradation of RNA molecules. They can temporarily be denatured under extreme conditions but it readily renatures. RNases can withstand temperatures up to 100° C, and extreme pH. It can survive autoclaving and other standard methods of protein inactivation. Therefore all materials used in extraction were kept free of RNase contamination.

3.2.1.1 Precautions to avoid RNase contamination

For the successful isolation of RNA and its further processing the following steps were taken.

1. Centrifuge tubes, micro tips, mortar, pestle, spatula and glassware were treated with 0.1 per cent DEPC, a strong inhibitor of RNases.
2. Sterile gloves were worn during the entire course of work to reduce RNase contamination.
3. The working area was cleaned with 70 per cent ethanol before starting the experiment.

4. Non-disposable plastic ware was rinsed in chloroform and washed with sterile DEPC treated water.
5. Electrophoresis trays and troughs were treated with 3 per cent hydrogen peroxide (v/v) for 10-15 minutes and then rinsed with RNase free water before the run.
6. DEPC treated autoclaved water was used to prepare all reagent solutions.

3.2.1.1.1 Preparation of DEPC solution

DEPC (0.1 per cent) solution was prepared by adding 1 ml of DEPC to one liter of distilled water and mixed well. The lab ware were then soaked in DEPC water in a separate autoclavable container, covered with aluminium foil and incubated in dark for 12 hours in an isolated area. After 12 hours of incubation, the DEPC treated water was poured off into a separate container. The treated lab ware were then rinsed several times with double distilled sterile water and autoclaved for 45 minutes on liquid cycle to remove the traces of DEPC. After autoclaving the equipments were baked for 10 hours at 60° C.

3.2.1.2 RNA ISOLATION PROTOCOLS

1. Protocol by Rubber research institute of India (RRII) Kottayam (Venkatachalam, et al., 1999)

Phenol based extraction method utilizes EDTA and SDS as RNase inhibitor and 3M LiCl for RNA precipitation.

2. Protocol by Schneitz (2000)

Utilize hot phenol for the isolation of RNA and 8M and 2M LiCl for RNA precipitation.

3. Protocol using Bangalore Genei RNA isolation kit

Utilize water saturated phenol for the isolation of RNA.

4. TRIZOL method

The reagent is a mono-phasic solution of phenol and guanidine isothiocyanate to minimize the problem of RNases and the use of isopropyl alcohol for the precipitation of RNA. The steps followed are given below.

3.2.1.2.2 RNA ISOLATION METHOD USING TRIZOL REAGENT

1. 100 mg of flower bud was weighed out and was taken in a DEPC treated, autoclaved mortar and pestle. The petals were cut into small pieces and ground into fine powder using liquid nitrogen.
2. The powder was mixed with 1 ml of TRIZOL reagent and is homogenized for five minutes at 28° C.
3. The homogenized sample was transferred to a 2 ml sterile micro centrifuge tube. To the sample 0.2 ml of chloroform was added, shaken vigorously by hand for 15 seconds and incubated for 3 minutes at 28° C.
4. The tubes were centrifuged at 12000 rpm for 15 minutes at 4° C.
5. The aqueous phase was carefully transferred in to a fresh tube without disturbing the lower layers and 500 µl.chilled isopropyl alcohol was added and incubated for 45 minutes at 28° C for precipitating RNA.
6. The tubes were centrifuged at 12000 rpm for 10 minutes at 4° C.
7. The supernatant was carefully removed and the gel like RNA pellets was washed two times with 75 per cent ethanol by centrifugation at 7500 rpm for 5 minutes at 4° C. The pellets were dried for 10 minutes.
8. The dried pellets were dissolved in 100 µl of RNase free DEPC water and incubated at 60° C for 10 minutes for the complete dissolution of the pellets. The isolated RNA was stored at –80° C.

3.2.1.3 QUALITATIVE AND QUANTITATIVE ANALYSES OF RNA

3.2.1.3.1 Qualitative analysis

The quality of isolated RNA was checked by agarose gel electrophoresis which is carried out in a horizontal gel electrophoresis unit (Genei, Bangalore).

1. 1.4 g of agarose was weighed out and dissolved in 1X TAE buffer (Appendix I) by boiling.
2. The solution was cooled to about 50° C and ethidium bromide was added to a final concentration of 0.5 µg/ml.
3. The mixture was transferred to a pre set template with appropriate comb.
4. After solidification of the agar the comb and the sealing tapes were removed and the gel was mounted in an electrophoresis unit.
5. The tank was loaded with 1X TAE buffer so that it just covered the entire gel.
6. Required volume of RNA sample and gel loading buffer (0.25 per cent bromophenol blue, 30 per cent glycerol, 70 per cent sterile water) were mixed.
7. Each well was loaded with 8 µl of sample.
8. Electrophoresis was performed at 75 volts until the loading dye reached ¾ length of the gel for 5 hours.
9. The gel was visualized using a gel documentation system (BIO-RAD, USA).

3.2.1.3.2 Quantitative analysis

UV spectroscopy was used for assessing the concentration of RNA. The absorbance of diluted samples was measured at 260 and 280 nm (SPECTRONIC GENESYS 5). The RNA concentration was calculated using Beer-Lamberts equation.

According to this an absorbance value of 1, at 260 nm corresponds to approximately 40 µg/ ml for RNA, the RNA concentration in the sample was calculated as follows

Concentration of RNA (µg/ml) =A_{260} x 40 x dilution factor

A_{260} = Absorbance at 260 nm

The purity of RNA was determined from the ratio between A_{260} and A_{280} readings.

3.2.2 mRNA PURIFICATION

Purification of mRNA from total RNA was carried out using mRNA purification kit (KT 80, Bangalore GENEI). The reaction is based on the batch binding of the polyadenylated mRNA with pre-measured quantities of Oligo (dT) $_{25}$ silica. The steps were as follows.

1. Binding

1. 100 µl of RNA (prepared as mentioned in section 3.2.1.2.2) was added to oligo d(T) $_{25}$ silica vials and mixed well by gentle tapping. The volume was made to 200 µl with DEPC water.
3. The tube was incubated at 65° C for five minutes with intermittent mixing.
4. 20 µl of 5M NaCl was added to the vial and incubated at 37° C for 10 minutes.
5. After 10 minutes of incubation the tube was centrifuged at 10,000 rpm for ten minutes at room temperature. The supernatant was carefully removed with micropipette without disturbing the pellet.

2. Washing

1. 200 µl of wash buffer was added to the pellet. The pellet was suspended by gentle tapping.
2. Centrifuged at 10,000 rpm for 10 minutes at room temperature.
3. Using a micropipette the supernatant was removed without disturbing the pellet and the same step was repeated twice.

3. Elution

1. The washed pellet was suspended gently in 50 µl of DEPC water and incubated at 65° C for five minutes.

2. The suspension was transferred on to a spin column using micropipette. The column was placed in a sterile 1.5 ml vial and centrifuged at 5000 rpm for five minutes to recover mRNA. The recovered mRNA was stored at -80° C.

3.2.3 RT-PCR

RT-PCR is a sensitive technique for mRNA detection and quantification. Compared to the two other commonly used techniques for quantifying mRNA levels, Northern blot analysis and RNase protection assay, RT-PCR can be used to quantify mRNA levels from much smaller samples.

RT-PCR was carried out using the RT-PCR kit (Bangalore GENEI, KT-74) in a Programmable Thermal Controller PTC-100 (MJ Research). Intact and undegraded mRNA is very essential for successful first strand synthesis and subsequent PCR amplification. All materials used in the experiment were autoclaved prior to use.

3.2.3.2 Materials used for RT-PCR

1. Good quality mRNA
2. oligo $d(T)_{18}$ primer
3. RNase inhibitor
4. 5X RT buffer
5. 30mM dNTP mix
6. M-MuLV reverse transcriptase
7. 0.1M DTT
8. 10X PCR buffer
9. Taq DNA polymerase
10. Gene specific primers designed

3.2.3.3 First strand synthesis

cDNA was prepared from 100 ng of RNA, which was reverse transcribed by M-MuLV reverse transcriptase (Genei, Bangalore) using oligo d $(T)_{18}$ primer. The reaction was carried out as follows.

1. 2 µl of RNA sample (100 ng/ µl) was added to a sterile PCR tube.
2. The volume was made to 9 µl with nuclease free water.
3. The reaction was mixed well by gentle tapping and 1µl oligo d $(T)_{18}$ primer was added to the mixture.
4. The vials were placed at 65° C for ten minutes and then at room temperature for two minutes. A brief spin was given to mix the contents.
5. To the reaction mixture the following reagents were added

ITEM	VOLUME (µl)
RNase inhibitor	1.0
0.1 M DTT	1.0
RT buffer (5X)	4.0
30mM dNTP mix	2.0
Reverse Transcriptase	0.5
Sterile water	1.0

6. The solution was mixed well and incubated at 37° C for one hour. After one hour the tubes were taken from water bath and kept at 95° C for two minutes to denature RNA-cDNA hybrids and immediately kept on ice.

3.2.3.4 PCR amplification

The cDNA was amplified using gene specific primers. 25 µl of reaction mixture was set in the programmable thermal controller PTC-100 (MJ Research, USA). The reaction was set as follows.

Item	Volume (µl)
cDNA product	3.0
10x PCR buffer	2.5
30mM dNTP mix	0.5
Forward primer (100 ng/µl)	0.5
Reverse primer (100 ng/µl)	0.5
Taq DNA polymerase(1U/µl)	1.0
Sterile water	7.0
Total	**25.0**

3.2.3.4.1 PCR conditions

Amplification of cDNA was carried out using the following programme.

Initial Denaturation:	95° C – 2.00 min	
	94° C – 45.0 sec	30 cycles
	52° C – 30.0 sec	
	72° C - 1.00 min	
Final extension	72° C - 2.00 min	

3.2.3.5 Electrophoresis of PCR product

After PCR cycles, gel electrophoresis was carried out on 1.2 per cent low melting agarose gel in 1X TAE buffer at 65 V for three hours. The amplified product was

loaded by mixing with the required volume of the gel loading buffer and run along with 1.0 µl of 100 bp DNA molecular weight marker.

3.2.4 CLONING OF PCR PRODUCT

The amplified products generated by PCR were ligated to pGEM-T Easy Vector using T4 DNA ligase and transferred to competent *E. coli* strain DH5α.

The pGEM-T Easy Vector contains multiple restriction sites within the multiple cloning regions flanked by recognition sites for the restriction enzymes *EcoRI*, *Bst ZI* and *NotI*, thus providing three single-enzyme digestions for release of the insert. The vector contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the alpha-peptide coding region of the enzyme β-galactosidase. The insertional inactivation of the α-pepetide allows recombinant clones to be directly identified by colour screening on indicator medium (Fig. 4 and 5). The transformants were analyzed by blue white screening and recombinants were selected to isolate plasmid DNA. The recombinant plasmid was used for sequencing.

3.2.4.1 Materials used for cloning

1. pGEM-T Easy Vector
2. 2X Rapid ligation buffer
3. PCR product
4. T4 DNA Ligase

3.2.4.2 Ligation of PCR product to pGEM-T Easy Vector

1. The tubes containing pGEM-T Easy Vector (50 ng) and 2X rapid ligation buffer tubes were briefly centrifuged to collect contents at the bottom of the tubes.
2. A ligation reaction was set up by adding the following.

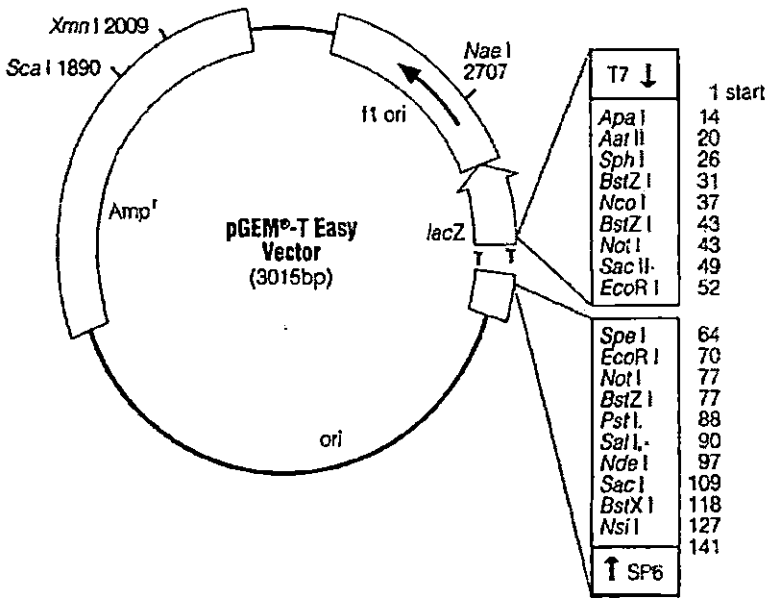

Fig. 4. pGEM-T Easy Vector circle map sequence reference points

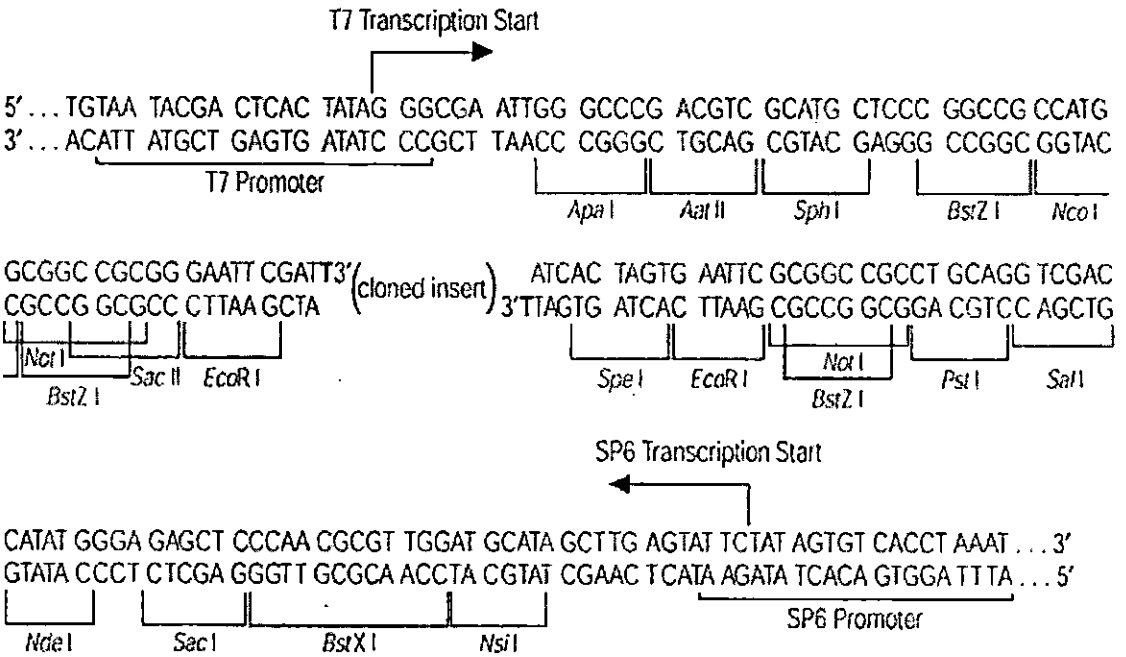

Fig. 5. The promoter and multiple cloning sequence of the pGEM-T Easy Vector

ITEM	Volume (µl)
2X Rapid ligation buffer	5.0
pGEM-T Easy Vector (50ng)	1.0
PCR product	2.0
T4 DNA Ligase (3U/µl)	1.0
Deionized water	1.0

3. After proper mixing the reaction mixture was incubated overnight at 4° C to get maximum number of transformants.

Volume of PCR product needed was calculated using the formula

$$\text{X ng insert:} \quad \frac{\text{ng of vector x kb size of insert}}{\text{Kb size of vector}} \quad \text{X} \quad \text{insert: vector molar ratio}$$

3.2.4.3 Preparation of competent cells

The *E. Coli* strain DH 5α used for transformation were made competent by the following treatments

1. Single cell colonies grown on Luria Bertani (L.B) agar plates were transferred in to 100 ml of L.B broth in a one liter flask and incubated at 37° C for 3 hours with vigorous shaking (300 rpm in a rotary shaker).
2. 30 ml of culture was transferred to a sterile 50 ml polypropylene tube and kept in ice for 10 minutes.
3. The cells are recovered from the culture by centrifugation at 4000 rpm for 10 minute at 4° C.
4. After centrifugation the media was decanted and the pellets were recovered.

5. The pellet was resuspended in 10 ml of ice-cold 0.1 M Calcium Chloride ($CaCl_2$) and kept in ice. The tubes were centrifuged at 4000 rpm for 10 minutes at 4° C.
6. The fluid was decanted from the cell pellets and resuspended in 2 ml of ice cold 0.1 M $CaCl_2$.
7. Using a chilled sterile pipette tip, 200 µl of competent cell suspension was transferred to a sterile 1.5 ml micro centrifuge tubes.

3.2.4.4 Transformation of DH5α Cells

1. L.B. plates containing 100µg/ml ampicillin, 100 mM Isopropyl-beta-thio-galactopyranoside (IPTG) and 50 mg/ml 5-Bromo-Chloro-3-Indolyl-β-D-Galactopyranoside (X-Gal) plates were prepared and stored ahead of the work.
2. The tubes containing the ligation reaction (mentioned as in section 3.2.4.2) was centrifuged to collect the contents at the bottom of the tube.
3. 2 µl of ligation reaction mixture was transferred to a sterile 1.5 ml micro centrifuge tube kept on ice.
4. The competent cells were taken from storage and kept on ice bath for 5 minutes to get thaw. The cells were mixed well.
5. 50 µl of cells were taken and carefully transferred to tubes containing 2 µl of ligation reaction.
6. The contents were mixed well and kept in ice for 20 minutes.
7. After 20 minutes the tubes were kept in a water bath (42° C for 45 seconds) to heat shock the cells.
8. After the prescribed time the tubes were immediately transferred to ice for 20 minutes.
9. 950 µl of SOC medium (Appendix II) was added and incubated for 1.5 hours at 37° C with shaking (150 rpm).
10. 100 µl of each transformation culture was plated on to LB/ampicillin/IPTG/X-Gal plates.
11. The plates were kept for overnight incubation (16 hours) at 37° C.

12. Next day the transformants were analyzed by blue white screening. Transformed white colonies were selected for isolating the plasmid DNA.

3.2.4.5 Plasmid DNA isolation protocol (Birnboim and Doly, 1979)

Each white colony was picked separately for isolating plasmid DNA. The colonies were grown overnight in 2 ml of LB broth containing 100 µg/ml ampicillin. The tubes were kept in a rotory shaker (ROTEK-LES) at 37° C for 12 hrs.

1. 1.5 ml of fresh culture was poured in to 2 ml micro centrifuge tube and spun at 10,000 rpm for one minute at 4° C.
2. The supernatant was removed completely without disturbing the pellet.
3. The pellet was resuspended in 100 µl ice cold solution I (Appendix III).
4. 200 µl of freshly prepared solution II (Appendix III) was added to the mixture and contents were mixed well by inverting the tubes rapidly five times.
5. The tubes were then stored on ice.
6. 150 µl of ice cold solution III (Appendix III) was added to the tubes. The tubes were vortexed gently in an inverted position for ten seconds and stored on ice.
7. After five minutes of incubation the mixture was centrifuged at 10,000 rpm for 5 minutes at 4° C.
8. The supernatant was then transferred to a fresh tube and the double stranded DNA was precipitated by adding two volumes of absolute ethanol at room temperature.
9. The tubes were centrifuged at 10,000 rpm for ten minutes at 4° C.
10. The pelleted DNA was then rinsed with 70 per cent ethanol; air dried and dissolved in 50 µl 10mM Tris Hydrochloride (Tris-HCl) (pH 8). The DNA was stored at -20° C.

The plasmid DNA was analyzed by agarose gel (1.2 per cent) electrophoresis. PCR amplification of the plasmid DNA was carried out using gene specific primer with same PCR conditions mentioned in section 3.2.3.4.1.

3.2.5 SEQUENCING OF CLONES

Sequencing was done at Chromous Biotech Pvt. Ltd. Bangalore. Automated sequencing using fluorescent labeled dye terminators are used to sequence the product. As the pGEM-T vector carries T7 and SP6 RNA polymerase promoters flanking a multiple cloning region, primers complementary to these sequences were used in the sequencing PCR reaction.

Results

4. RESULTS

The salient findings of the study entitled "Isolation and characterization of cDNA encoding dihydroflavonol 4-reductase gene from orchid *Dendrobium* variety Sonia 17" conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram are presented below.

4.1 Primer designing

Using Primer 3 software, three primer sets were designed from *dfr* cDNA sequence of *Cymbidium* orchid (Primer set Af, Ar and Cf, Cr) and *Oryza sativa* (Bf, Br). Primer set Df and Dr was designed from the heterologous dihydroflavonol 4-reductase gene sequences from other crops using the bioinformatics tools. Complementary DNA sequences of *dfr* gene of different species were downloaded from NCBI in FASTA format. The gene identity and the source plants used are shown in Table 1. Using Clustal X 1.81 multiple alignment programme the homologous regions of the gene were identified (Fig 6). Based on the conserved sequences, forward and reverse primers were designed using Primer 3 software. The best primer combination for the gene was identified based on annealing temperature optimal for both forward and reverse primers, presence of minimum number of mononucleotide repeats, minimum 3 ' (tendency to form primer-dimer) and any (tendency to form secondary structure) complementarity values (Table 2). BLASTN analysis of designed primer showed the binding specificity of the primer (Fig 7).

Primers designed based on the dihydroflavonol 4-reductase sequences obtained from *Cymbidium* orchid and *Oryza sativa* was selected for amplifying the cDNA encoding *dfr* gene from *Dendrobium* Sonia 17 orchids.

Table 1. Details of *dfr* gene sequences used for designing gene specific primer D

Sl. No	Gene identity	Source
1	DQ 421810	*Anthurium andraeanum*
2	AF 134807	*Oryza sativa* (Japonica cultivar)
3	Z 17221	*Gerbera hybrida*
4	EF570113	*Oncidium* Gower Ramsey
5	AY 373831	*Triticum aestivum*

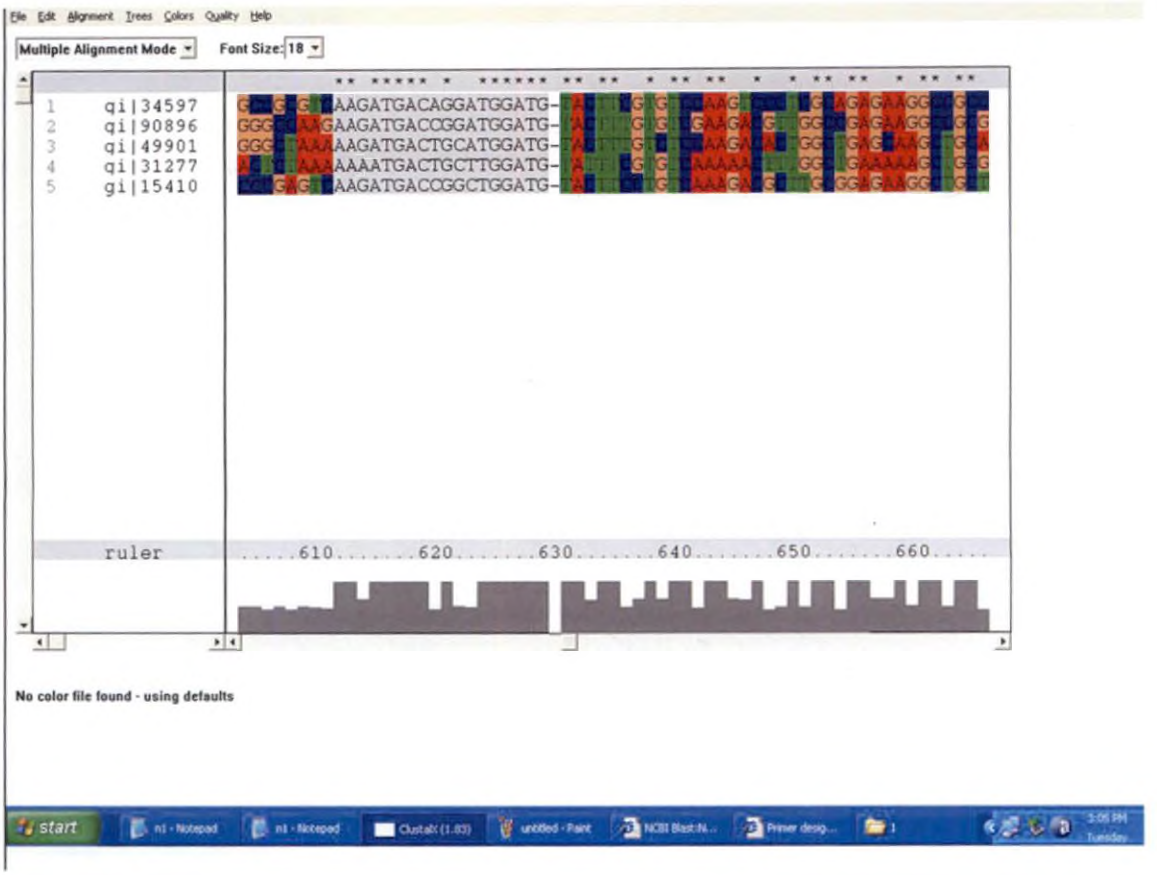

Fig.6: Input form for Clustal X 1.81 multiplen alignment programme for the Primer D

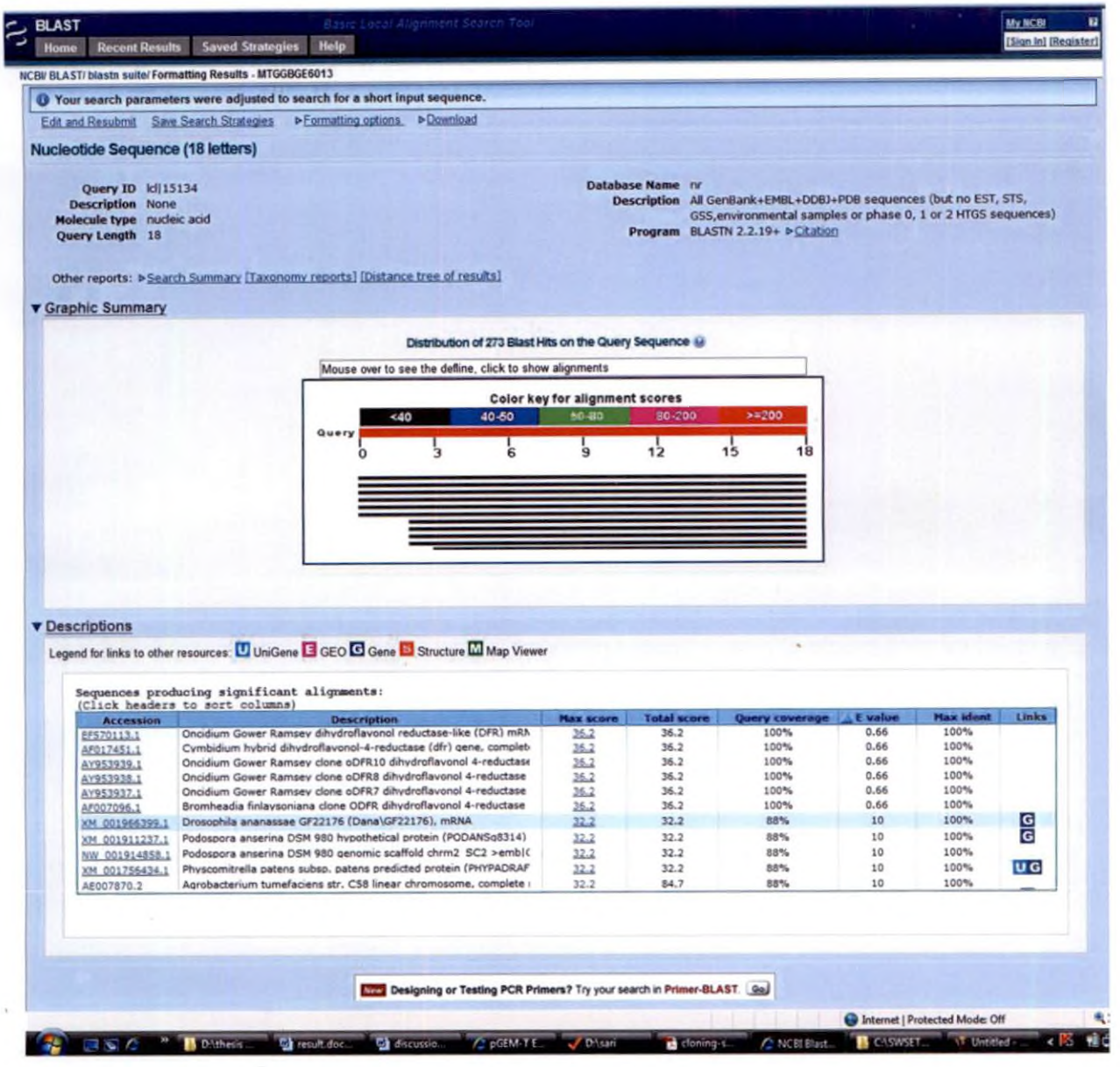

Fig.7: BLASTN result for the Primer set D

Sl. No	Name of Primer	Tm (° C)	GC%	Primer length (nucleotide)	Sequence	Source plant	Expected amplicon size
1	Af Ar	59.86 60.29	57.14 52.38	21 21	AGACTGAGAGGAAGGGTCCAG GAGAAGCTCACGCTCTCAATG	*Cymbidium hybrid*	500-800 bp
2	Bf Br	60.26 59.27	52.63 52.38	19 21	GCGAATCCAACACAAGCAC AGCGTGTACCTGAACCTGAAC	*Oryza sativa* (Japonica cultivar)	500-800 bp
3	Cf Cr	56.8 52.9	60.00 45.00	20 20	CTGAGAGGAAGGGTCCAGTG CTGCCATTTGCTTTGTGATG	*Cymbidium hybrid*	400-600 bp
4	Df Dr	59.58 60.07	55.56 50.00	18- 20	AAGATGACCGGCTGGATG CGGAGACCATTTCTCGGAT	*Anthurium andraeanum* *Oryza Sativa* *Gerbera hybrida* *Oncidium* gower *Ramsey* *Triticum aestivum*	500-800 bp

Table 2. Details of primers designed

4.2 Isolation of RNA

RNA was isolated from the flower buds. As the *dfr* gene shows its expression throughout the flower development buds of all the stages were used (Plate 1). Four protocols were tried. Protocols developed at Rubber Research Institute of India (RRII) Kottayam and the hot phenol method of RNA isolation by Schneitz, (2000) were not yielded good quality RNA in case of Sonia 17 floral tissues (Plate 2). The RNA obtained was degraded, the pellets were hard and showed difficulty in dissolving. The protocol using Genei RNA isolation kit yielded good quality RNA, but the loose gel like pellet showed problem in dissolving and the yield was less. However, the protocol using TRIZOL reagent yielded good quality intact RNA. The isolated RNA pellet was dissolved in 100 µl of DEPC treated sterile water and stored at -80° C. The purity of RNA was good with an A_{260}/A_{280} ratio ranging between 1.6-2.0.

The integrity and size distribution of total RNA was determined by running the RNA on 1.4 per cent agarose gel. The isolated RNA showed two intact bands corresponding to 18S and 28S rRNA with no genomic DNA contamination (Plate 3).

4.3 mRNA purification

For the separation of mRNA from other RNA fractions, purification of total RNA was done by mRNA purification kit of Bangalore Genei. The total RNA was purified following the instructions given by the manufacturer. The purified mRNA was dissolved in DEPC treated sterile water and stored at -80° C. The purified sample showed an absorbance value (A_{260}/A_{280}) of more than 1.8.

4.4 RT-PCR

Reverse transcription polymerase chain reaction was carried out for gene specific amplification. Using reverse transcriptase enzyme, cDNA was produced from purified mRNA following the instructions of manufacturer. Three primer pairs were used for the




Plate 1:Flower buds of *Dendrobium* Sonia 17 Orchid showing different stages

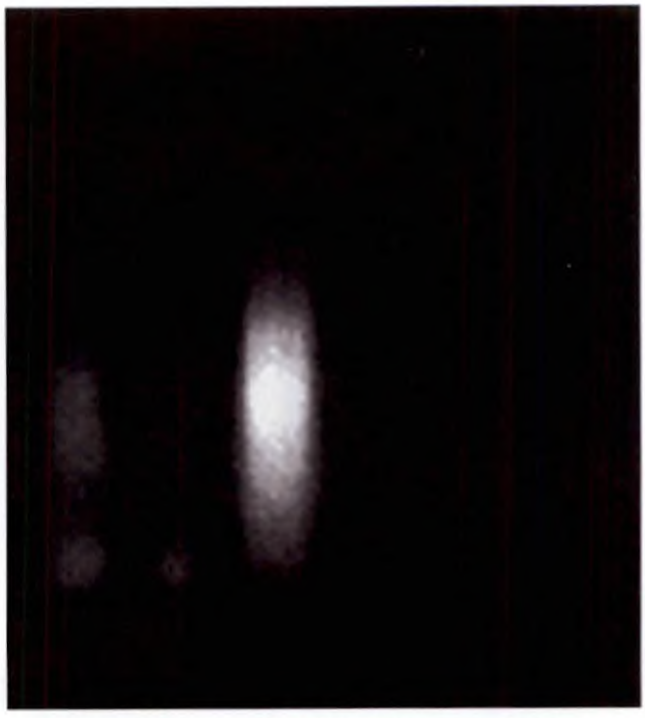

Plate 2: Total RNA extracted with the RRII extraction protocol

Plate 3 : Total RNA extracted with TRIZOL reagent

amplification (Primer Af, Ar, Bf, Br, and Cf, Cr). All the three primers produced reproducible amplification. The primer pair A produced an amplified product of size 400-500 bp. Primer pair B showed a band size of nearly 300 bp and that of C showed a band of size nearly 100 bp as seen on gel analysis (Plate 4)

Primer dimer formation was noticed during PCR reactions initially, which was indicated by a band of size nearly 50 bp on gel analysis. The dimer formation was reduced by optimizing the reaction like increasing the concentration of cDNA template and lowering primer concentration.

4.5 Cloning of PCR product

The amplified product obtained using the Primer Af and Ar was cloned to pGEM-T Easy Vector. The transformed vectors were multiplied in *E. Coli* ((DH5α) cells and the colonies were screened on plated on L.B agar plates supplemented with 100 mM IPTG, 50 mg/ml X-Gal and 100 µg/ml ampicillin. The colonies were analyzed by blue - white screening. The transformed cells produced small white colonies (Plate 5).

4.5.1 Plasmid DNA Isolation and PCR amplification

Three white colonies were randomly selected and grown in L.B broth containing 100µg/ml ampicillin. Plasmid DNA from the cultures was isolated by standard protocol. Agarose gel electrophoresis (1.2 per cent) of the plasmid DNA showed intact bands. The presence of PCR product was checked by PCR using primer set A. The products were analyzed on 1.2 per cent agarose gel (Plate 6). Gel analysis of the amplified plasmid DNA from the three clones showed amplification showing the presence of insert. However, more than one band was obtained.

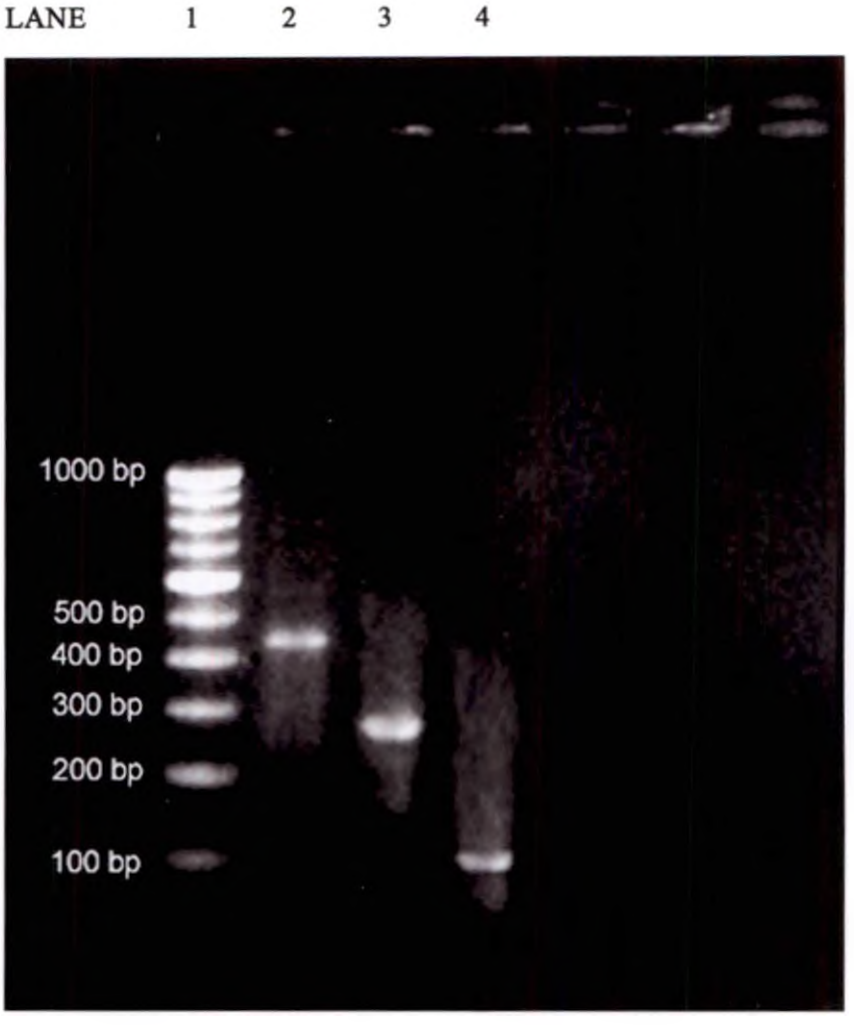

Plate 4 : cDNA amplified using dihydro flavonol 4- reductase specific primer in orchid

Lane 1 : 100bp DNA Marker
Lane 2 : cDNA amplified with primer Af and Ar
Lane 3 : cDNA amplified with primer Bf and Br
Lane 4 : cDNA amplified with primer Cf and Cr

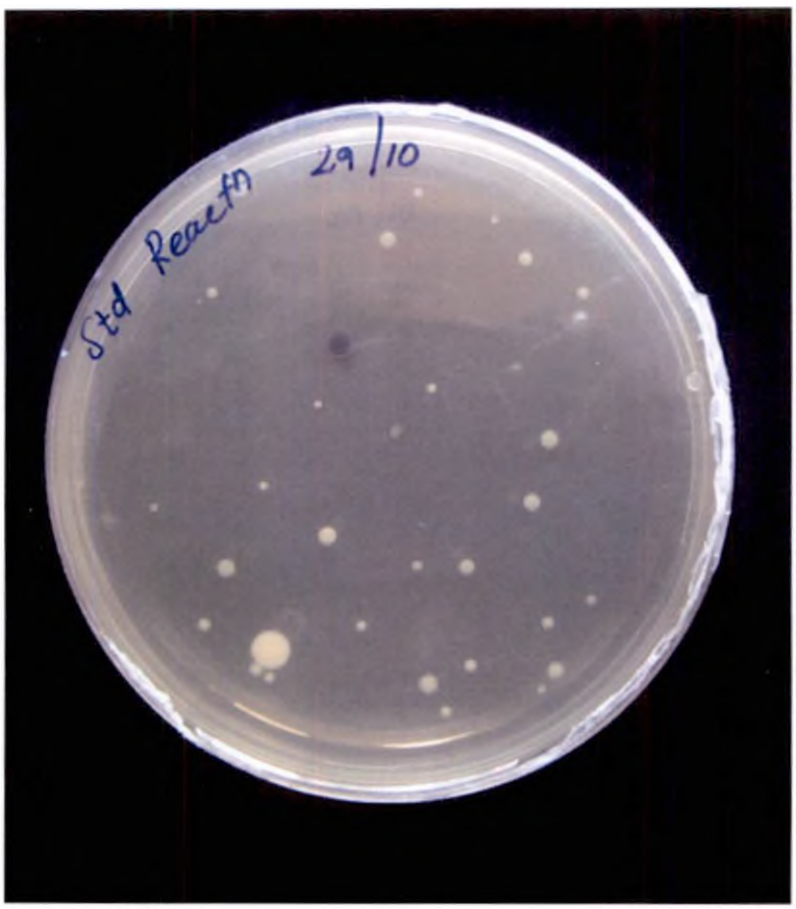

Plate 5: Transformed *E.Coli* colonies

White colony : Transformed

Blue colony : Non Transformed

LANE 1 2 3 4

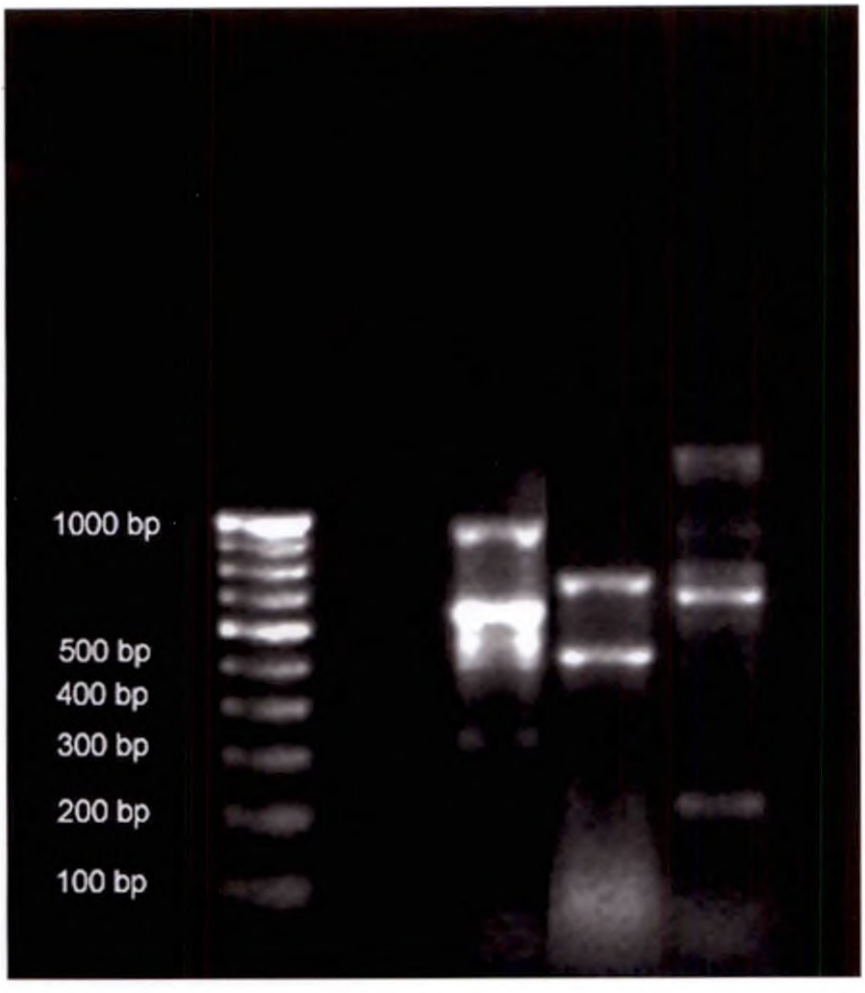

Plate.6 : PCR amplified product of plasmid DNA using gene specific Primar A

Lane 1 : 100 bp DNA marker
Lane 2 : Clone 1
Lane 3 : Clone 2
Lane 4 : Clone 3

4.6 SEQUENCING

Two clones were selected for sequencing. Sequencing was done at Chromous Biotech Pvt. Ltd. Bangalore. Since the vector contain T7 (ATTATGCTGAGTGATATC) and SP6 (TAAGATATCACAGTGGATTTA) promoter sites, these sequences were used as forward and reverse primer to amplify plasmid DNA. However, sequencing reaction using these primers failed to amplify the cloned PCR product.

Discussion

5. DISCUSSION

Orchids are one of the important cut flower crops noted for their beautiful long lasting flowers, widely differing in size, shape and colour. Orchid cut flower industry is highly developed, for local and export markets. Global demand for orchids is increasing. *Dendrobium,* one of the important cut flower orchid, is best suited to our conditions and the variety Sonia 17 is highly popular among the farmers of Kerala. Market value of orchid flowers is mainly determined by flower colour, architecture and vase life. Novel varieties are always in demand and new varieties constantly replace the existing ones. Flower colour is an important trait in orchid which is governed by the anthocyanin biosynthetic pathway. Any change in this pathway will lead to modification of flower colour. Metabolic engineering is an emerging technology widely used to modify specific traits by altering the key enzymes in the biosynthetic pathway. This technique has been used to modify flower colour in a few flower crops (blue/violet carnation and rose) which are already available in the market. Modification in these crops has been achieved by introducing genes from other sources or by regulating the existing genes in the biosynthetic pathway. In orchids no work has been reported so far using this technology to modify flower colour.

The spectrum of colours found in orchids is mainly due to the accumulation of anthocyanins in flowers (Arditti and Fisch, 1977). The anthocyanin biosynthetic pathway is very complex, involving large number of structural and regulatory genes. Alteration of the genes affects pigmentation (Jaakola, 2003). Factors affecting flower colour are genetically determined, primarily. In addition, environmental factors such as nutrients, temperature and light conditions can have an effect on anthocyanin and flavonoid composition.

For the regulation of biosynthetic pathway, understanding of the genes coding for the key enzymes is very essential. Dihydroflavonol 4-reductase is one of the key enzymes involved in the anthocyanin biosynthesis. DFR catalyzes the last common step

in the flavonoid biosynthesis pathway leading to anthocyanins and proanthocyanidins. Alteration of this gene to change the flower colour has been reported in rose, carnation and several other crops. However, there are no reports on the identification or manipulation of key structural and regulatory genes of biosynthetic pathway in *Dendrobium* Sonia 17. In view of these facts the present study was taken up focusing on the isolation and characterization of dihydroflavonol 4-reductase gene from *Dendrobium* Sonia 17, using Reverse Transcription - PCR.

Since, there is no report on the dihydroflavonol 4-reductase gene sequence in *Dendrobium* Sonia 17 orchids, gene specific primers were designed from the heterologous gene sequences of other crops. Three primer sets (primer Af, Ar; Bf, Br and Cf, Cr) were designed based on the *dfr* cDNA sequences collected from *Cymbidium* orchid and *Oryza sativa* using Primer 3 software (Rozen and Skaletsky, 2000). Primer pair Df and Dr were designed based on the multiple sequence alignment of *dfr* gene sequences, collected from other crop species. The homologous regions of the genes were identified using Clustal X 1.81 multiple alignment programme (Thomson et al., 1997) and these homologous sequences were used to design primer using Primer 3 software. The binding specificity of the designed primers was checked by BLASTN (Altschul et al., 1997) analysis. The BLASTN analysis of the designed primers showed homology with the sequences reported in orchid species like *Oncidium* and *Cymbidium*.

Flower buds of all the stages were used to isolate RNA, since *dfr* gene shows its expression throughout the flower development (Mudalige-Jayawickrama et al., 2005). Orchid floral tissues contain large amount of polyphenols and polysaccharides which interfere with the isolation of intact RNA. In addition, the presence of large amount of endogenous RNase and the deeply coloured nature of orchid floral tissues are also affect the quality of RNA. So, four protocols were tried to isolate intact RNA, free from polyphenols and polysaccharides. The protocol developed by the Rubber Research Institute of India, Kottayam for isolating RNA from bark tissues of *Hevea brasiliensis* (Venkatachalam et al., 1999) did not yield good quality RNA. Even though this protocol utilizes RNase inhibitors such as EDTA and SDS, it was not sufficient to inhibit the

RNase in orchid floral tissues which led to shearing of RNA. The pellets were too hard and showed difficulty in dissolving. Protocol by Schneitz, (2000) which utilizes hot phenol maintained at 80° C to avoid the problem of polyphenols also yielded degraded RNA. Protocol using Bangalore Genei RNA isolation kit, yielded good quality RNA, but there was some problem associated with the isolation procedure. The loose gel like RNA pellet showed difficulty in dissolving and the quantity was also less. TRIZOL based extraction protocol was found to be the best for getting good quality RNA. The pellet obtained by TRIZOL also showed dissolution problem; but it was less compared to the other methods. The TRIZOL reagent, a monophasic solution of phenol and guanidium isothiocyanate maintained the integrity of RNA and avoided the protein contamination and RNase contamination during isolation steps. Compared to other isolation protocols TRIZOL extraction protocol could be completed within a short period of time with comparatively good yield. The problem of residues of coloured substances which binds to RNA to form insoluble complexes (Pawlowski et al., 1994) could be overcome by a washing step with ethanol, after the precipitation of RNA with isopropyl alcohol.

Electrophoresis of RNA on 1.4 per cent agarose gel showed two clear bands corresponding to 18S and 28S rRNA, indicating that the isolated RNA was totally intact with no genomic DNA contamination.

Purified mRNA yields better results in reverse transcription, compared to total RNA (Aviv and Leder, 1972). The purified mRNA improves the efficiency of reverse transcription by providing more templates for reverse transcriptase to act. Hence, mRNA purification was carried out using mRNA purification kit of Bangalore Genei. The kit utilizes oligo d(T) silica to recover mRNA by specific binding with polyadenylated mRNA.

Reverse transcription PCR was carried out for cDNA amplification. From the four primer sets designed, three (primer set Af, Ar; Bf, Br and Cf, Cr) were selected for RT-PCR of the cDNA encoding *dfr* gene from Sonia 17. All the primers included in the study showed amplification. The PCR amplified products were gel electrophored on 1.2

per cent low melting agarose gel in 1X TAE buffer. The primer set Af and Ar, designed from *Cymbidium* orchid which was expected to amplify a product of size 500-800 bp, yielded a fragment of 400-500 bp. Primer set Bf and Br designed from *Oryza sativa dfr* cDNA sequences showed an amplified product of size nearly 300 bp and the expected size range was 500-800 bp. Primer set Cf and Cr, also synthesized from cDNA sequences of *Cymbidium* orchid with an anticipated product size of 400-600 bp, gave an amplified product of size nearly 100 bp. It was understood that all the heterologous primers designed were able to identify the homologous sequences of *dfr* gene in *Dendrobium* Sonia 17. The reaction conditions used for all primers were the same. An annealing temperature of 52° C was kept for all the primers as there was not much difference in the Tm of the primers.

The amplification reaction with all the three primers showed a band of nearly 50 bp on gel analysis indicating the formation of primer dimer. There are chances for the formation of primer dimers when the reaction mixture has low annealing temperature, higher primer concentration and low concentration of template (Brownie et al., 1997). Hence the reaction conditions were optimized by providing a higher template concentration and low primer concentration. By increasing the template cDNA concentration the dimer formation could be reduced.

Only the amplified product obtained using the primers Af and Ar was sequenced. The PCR product was cloned to pGEM-T Easy Vector purchased from Promega Corporation. The pGEM-T Easy Vector multiple cloning regions is flanked by recognition sites for the restriction enzymes *EcoR*I, *BstZ*I *and Not*I provides three single-enzyme digestions for the release of the insert. The vector also contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows selection of recombinant clones by colour screening on indicator plates. The recombinant vectors were multiplied in competent *E. Coli* cells (DH5α) on L.B broth.

The transformed cultures were plated on LB agar plates supplemented with 100mM IPTG, 50 mg/ml X-Gal and 100 µg/ml ampicillin. Overnight incubation at 37°C produced blue and white colonies. The transformed colonies were seen as small and white. The colonies appeared white because of the insertional inactivation of the coding sequences of β-galactosidase by the insert DNA.

From the transformed colonies three were randomly selected for sequencing. Single colonies were transferred into LB broth for growth and the plasmid DNA was extracted by standard protocol (Birnboim and Doly, 1979). The integrity of DNA was checked by gel analysis. To ensure the presence of amplified product as the insert, PCR amplification of plasmid DNA was carried out with gene specific primers Af and Ar. Gel analysis (1.2 per cent agarose) of the amplified plasmid DNA from the three clones showed amplification showing the presence of insert. However, more than one band was obtained. This might be due to the degradation of cDNA amplified product and insertion of fragments of different size in to the vector DNA.

Two of the colonies were selected for sequencing. The authorized service facility of Chromous Biotech Pvt. Ltd was used for the purpose. For the sequencing reaction T7 and SP6 promoter sequences, were used as forward and reverse primers to amplify the plasmid DNA. However, the sequencing reaction failed to amplify the plasmid DNA by these primers. The problem might be due to the reaction conditions provided or escape of the insert during the procedure. The procedure may be repeated with other clones to get the sequences of cDNA encoding *dfr* gene.

This work was an initial step towards isolation and characterization of dihydroflavonol 4-reductase gene in *Dendrobium* variety Sonia 17. As this gene codes one of the key enzymes in the biosynthesis of anthocyanin, information regarding this can be utilized in the improvement of flower pigmentation in *Dendrobium* orchids.

Summary

6. SUMMARY

The study entitled "Isolation and characterization of cDNA encoding dihydro flavonol 4-reductase gene from *Dendrobium* orchid variety Sonia 17" was conducted at the Department of Plant Biotechnology College of Agriculture Vellayani, Thiruvananthapuram during 2006-2008. The study focused on designing primer for *dfr* gene, amplification of cDNA encoding *dfr* and its sequencing. Inflorescence of Sonia 17 orchids was subjected to analysis. Since, the *dfr* gene shows its expression throughout the flower development all the stages of flower buds were used. The salient findings of the study are summarized below.

RT-PCR technique was used for the isolation of *dfr* gene. Heterologous primers were designed based on the cDNA sequences of *dfr* gene reported in other crop species. Using Clustal X 1.81 multiple alignment programme homologous regions of the selected cDNA sequences were identified and the primers were designed using Primer 3 software. Primers were also designed based on the individual cDNA sequences derived from *Cymbidium* orchid and rice using Primer3 software.

Extraction of RNA from flower tissues was difficult due to the presence of large amounts of polyphenols and polysaccharides. Four protocols were tried in this study. The extraction protocol utilizing TRIZOL reagent was the best for isolating RNA from Sonia 17 floral tissues. The isolated RNA, analyzed by 1.4 per cent agarose gel, showed two intact bands corresponding to 28S and 18S rRNA with no genomic DNA contamination. Spectrophotometric measurements (A_{260}/A_{280}) of the isolated RNA indicated good quality of the sample.

cDNA was amplified using gene specific primers designed. The primers designed based on the *dfr* gene sequence of *cymbidium* orchid (Primer A and C) and *Oryza sativa* (Primer B) were used for gene specific amplification. The primer Af and Ar produced a

fragment of size 400-500 bp. The primet sets B (Bf and Br) and C (Cf and Cr) produced fragments of size approximately 300 bp and 100 bp respectively.

The amplified product of primer A was cloned in Promega pGEM-T Easy Vector, multiplied in competent *E. Coli* strain DH5α. and sequenced. The *E. Coli* colonies were grown on L.B agar plates supplemented with 100mM IPTG, 50 mg/ml X-Gal and 100 µg/ml ampicillin. The transformed colonies were analyzed by blue - white screening. Three white colonies were randomly selected, grown in L.B broth and the plasmid DNA was isolated using standard protocol. The presence of the amplified fragment was confirmed by PCR amplification of the plasmid DNA using gene specific primer A. Gel analysis showed amplification, however, all the colonies yielded more than one amplified products.

Two colonies were used for sequencing. As the vector processes T7 and SP6 promoter sequences, these sequences were used as primers in the sequencing PCR reaction. Authorized service facility of Chromous Biotech Pvt. Ltd. was used for this purpose. Automated sequencing using fluorescent labeled dye terminators was used to sequence the clones. However, the sequencing reaction using T7 and SP6 specific primers failed to amplify the plasmid DNA.

This work was an initial step for the isolation and characterization of the key anthocyanin biosynthetic gene, dihydroflavonol 4-reductase from *Dendrobium* Sonia 17. The primers designed in this study could successfully amplify the cDNA encoding *dfr*. The sequencing reaction need to be repeated with other clones.

References

REFERENCES

Ainsworth, C. 1994. Isolation of RNA from floral tissues of *Rumex acetosa* (Sorrel). Pl. Mol. Biol. Rep. 12: 198-203

Albertsen, C., Kalland, K. H., Haukanes, B. I., Havarstein, L. S. and Kleppe, K. 1990. Applications of magnetic beads with covalently attached oligonucleotide in hybridization: Isolation and detection of specific measles virus mRNA from a crude cell lysate. Anal. Biochem. 189: 40-50

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402

Anzai, H., Ishii, Y., Schichinohe, M., Katsumuta, K., Nojiri, C., Morikawa, H. and Tanaka, M. 1996. Transformation of *Phalaenopsis* by particle bombardment. Pl. Tissue Cult. Lett. 13: 265-272

Arditti, J. and Fisch, M. H. 1977. Anthocyanins of the Orchidaceae: Distribution, Heridity, Functions, Synthesis, and Localization. In: Arditti, J. (ed.). Orchid Biology - Reviews and Perspectives, I. Cornell University Press Ltd, London, UK, p. 117-155.

Asen, S., Stewart., R. N. and Norris, K. H. 1979. Pelargonidin 3-di (p-hydroxybenzoyl) rutinoside -7-glucoside from flowers of *Campanula*. Phytochemistry 18: 1251-1252

Asif, M. H., Dhawan, P. and Nath, P. 2000. A simple procedure for the isolation of high quality RNA from ripening banana fruit. Pl. Mol. Bio. Rep. 18: 109-115

Aslanidis, C. and de Jong, P. J. 1990. Ligation – independent cloning of PCR products (LIC-PCR). Nucleic Acids Res. 18: 6069-6074

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. 1987. Current Protocols in Molecular Biology. John Wiley and Sons, New York, p.1865

Avila, C. and Canovas, F. M. 2000. An improved and rapid protocol for the isolation of poly(A^+) - RNA from small samples of scots pine seedlings. Pl. Mol. Biol. Rep. 18: 117-122

Aviv, H. and Leder, P. 1972. Purification of biologically active globin messenger RNA by chromatography on oligo thymidylic acid - cellulose. Proc. Nat. Acad. Sci. 69: 1408

Bank, A., Terada, M., Metatora, S., Dow, L. and Marks, P. A. 1972. *In vitro* synthesis of DNA components of human genes for globins. Nat. New. Biol. 231: 167-169

Batschauer, A., Ehmann, B. and Schafer, E. 1991. Cloning and characterization of a chalcone synthase gene from mustard and its light - dependent expression. Pl. Mol. Biol. 16: 175-185

Bernhardt, J., Stich, K., Schwarz, Z., Saedler, H. and Wienand, U. 1998. Molecular analysis of a second functional *A1* gene (dihydroflavonol 4- reductase) in *Zea mays*. Pl. J. 14: 483-488

Biran, I. and Halevy, A. H. 1974. Effect of varying light intensities and temperature treatments applied to whole plants, or locally to leaves or flower buds, on growth and pigmentation of 'Baccara' roses. Physiol. Pl. 31: 175-179

Birnboim, H. C. and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513

Bohm, B. 1998. Introduction of Flavonoids. Harwood Academic Publishers, Singapore. p. 345

Bowler, C., Neuhaus, G., Yamagata, H. and Chua, N. H. 1994. Cyclic GMP and calcium mediate phytochrome signal transduction. Cell 77: 73-81

Boyd, A. C. 1993. Turbo cloning: A fast, efficient method for cloning PCR products and other blunt ended DNA fragments in to plasmids. Nucleic Acids Res. 21: 817-821

Brouillard, R. and Dangles, O. 1994. Flavonoid and flower colour. In: Harborne, J. B. (ed.). The Flavonoids: Advances in Research since 1986. Chapman and Hall, London, p. 565-588.

Brownie, J., Shawcross, S., Theaker, J., Whitcombe, D., Ferrie, R., Newton, C. and Little, S. 1997. The elimination of primer-dimer accumulation in PCR. Nucleic Acids Res. 25: 3235-3241

Brugliera, F., Tull. D., Holton T. A., Karan. M., Treloar. N., Simpson. K., Skurczynska. J., and Manson. J. G. 2000. Introduction of a cytochrome b5 enhances the hydroxylase (a cytochrome p450) in transgenic carnation. Sixth International Congress of Plant Molecular Biology. University of Laval, Quebec, p.6-8.

Chan, Y. L., Chen, W. H. and Chan, M. T. 2005. *Phalaenopsis* Orchid Gene Transformation (I) – Optimization of Transient Gene Expression. J. Chinese. Soc. Hort. Sci. 49: 33-44

Chang, S., Puryear, J. and Cairney, J. 1993. A simple method for isolating RNA from pine trees. Pl. Mol. Biol. Rep. 11: 113-116

Charrier, B., Coronada. C., Kondorosi. A. and Ratet, P. 1995. Molecular characterization and expression of alfalfa (*Medicago sativa* L.) flavanone-3-hydroxylase and dihydroflavonol 4 - reductase encoding genes. Pl. Mol. Biol. 29: 773-786

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G. and Thompson, J. D. 2003. Multiple sequence alignment with the clustal series of programs. Nucleic Acids Res. 31: 3497-3500

Chia, T. F., Chan, Y. S. and Chua, N. H. 1994. The firefly luciferase gene as a non-invasive reporter for *Dendrobium* transformation. Pl. J. 6: 441-446

Chirgwin, J. M., Przybyla, A. E., Mcdonald, R. J. and Ratter, W. J. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. Biochemistry 18: 5294-5299

Chomczynski, P. 1992. Solubilization in formamide protects RNA from degradation. Nucleic Acids Res. 20: 3791

Chomczynski, P. and Sacchi, N. 1987. Single – step method of RNA isolation by acid guanidium thiocyanate - phenol - chloroform extraction. Anal. Biochem. 162: 156-159

Christensen, K. I. and Hansen, H. V. 1998. SEM - studies of epidermal patterns of petals in the angiosperms. Op. Bot. 135: 5-86.

Clark, J. M. 1988. Novel non - templated nucleotide addition reactions catalyzed by prokaryotic and eukaryotic DNA polymerases. Nucleic Acids Res. 16: 9677-9686

Clement, J. S. and Mabry, T. J. 1996. Pigment evolution in the Caryophyllales: a Systematic Overview. Bot. Acta. 109: 360-367

Cox, R. A. 1968. The use of guanidium chloride in the isolation of nucleic acids. Methods Enzymol. 12: 120

Das, O. P., Alvarez, C., Chaudhuri, S. and Messing, J. 1990. Molecular methods for genetic analysis of maize. Methods Mol. Cell Biol. 1: 213-222

Davies, K. M. and Schwinn, K. E. 1997. Flower colour. In: Geneva, R. L., Preece, J. E and Merkle, S. A. (ed.). Biotechnology of Ornamental Plants, CAB International, Wallingford, UK. p. 259-294.

Davies, K. M., Bloor, S. J., Spiller, G. B. and Deroles, S. C, 1998. Production of yellow colour in flowers: redirection of flavonoid biosynthesis in petunia. Pl. J. 13: 259-266

Davies, K. M., Schwinn, K. E., Deroles, S. C., Manson, D. G., Lewis, D. H., Bloor, S. J. and Bradley, J. M. 2003. Enhancing anthocyanin production by altering competition for substrate between flavonol synthase and dihydroflavonol 4-reductase. Euphytica. 131: 259-268

Dressler, R. L. 1990. The orchids: natural history and classification, 2nd ed. Harvard Univ. Press, Cambridge, Mass

Durbin, M. L., Learn, G. H., Huttley, G. A., and Clegg, M. T. 1994. Evolution of the chalcone synthase gene family in the genus *Ipomoea*. Proc. Natl. Acad. Sci. USA. 92: 3338-3342

Edmonds, M., Vaugham, M. H. and Nakazato, H. 1971. Polyadenylic acid sequences in the heterogenous nuclear RNA and rapidly labeled polyribosomal RNA of HeLa cells: Possible evidence for a precursor relationship. Proc. Nat. Acad. Sci. *USA*. 68: 1336

Ellestad, G. A. 2006. Structure and chiroptical properties of supramolecular flower pigments. Chirality 18: 134-144

Elomaa, P. and Holton, T. A. 1995. Modification of flower colour using genetic engineering. Biotech. Genetic Eng. Rev. 12: 63-88

Farrel, R. E. 1998. RNA methodologies: A laboratory guide for isolation and characterization, 3rd ed. Academic Press, San Diego, p. 110-111

Fedorcsak, I. and Ehrenberg, L. 1966. Effects of diethyl pyrocarbonate and methyl methanesulfonate on nucleic acids and nucleases. Acta. Chem. Scand. 20: 107

Forkmann, G. and Ruhnau, B. 1987. Distinct substrate specificity of dihydroflavonol 4-reductase from flowers of *Petunia hybrida*. Z. Naturforsch, 42c: 1146-1148.

Forkmann, G. 1991. Flavonoids as flower pigments: The formation of the natural spectrum and its extension by genetic engineering. Plant Breed. 106: 1-26.

Forkmann, G. and Heller, W. 1999. Biosynthesis of flavonoids. In: Sankawa, U. (ed.) Polyketides and Other Secondary Metabolites Including Fatty Acid and Their Derivatives. Elsevier, Amsterdam, p. 713–748.

Forkmann, G. and Martens, S. 2001. Metabolic engineering and applications of flavonoids. Curr. Opin. Biotechnol. 12: 155-160

Fukusaki, E., Kawasaki, K., Kajiyama, S., An, C. I., Suzuki, K., Tanaka, Y. and Kobayashi, A., 2004. Flower colour modulations of *Torenia hybrida* by down regulation of chalcone synthase gene with RNA interference. J. Biotechnol. 111: 229-240

Gehring, H. H., Winter, K., Cushman, J., Borland, A. and Taybi, T. 2000. An improved RNA isolation method for succulent plant species rich in polyphenols and polysaccharides. Pl. Mol. Biol. 18: 369-376

Gerard, G. F., Fox, D. K., Nathan, M. and D' Alessio, J. M. 1997. Reverse Transcriptase: The use of cloned Mu MLV- RT to synthesize DNA from RNA. Mol. Biotechnol. 8: 61-77

Glover, B. J. 2000. Differentiation in plant epidermal cells. J. Expt. Bot. 51: 497-505.

Gollop, R., Even, S. Colova – Tsolova, V. and Per, A. 2002. Expression of the grape dihydroflavonol 4-reductase gene and analysis of its promoter region. J. Exp. Bot. 53: 1397-1409

Gong, Z., Yamazaki, M., Sugiyama, M., Tanaka, Y. and Saito, K. 1997. Cloning and molecular analysis of the structural genes involved in anthocyanin biosynthesis and expressed in a forma-specific manner in *Perilla frutescens*. Pl. Mol. Biol. 35: 915-927

Gorton, H. J. and Vogelmann, T. C. 1996. Effect of epidermal cell shape and pigmentation on optical properties of *Antirrhinum* petals at visible and ultraviolet wavelengths. Pl. Physiol. 112: 879-888.

Goto, T. and Kondo, T. 1991. Structure and molecular stacking of anthocyanins - flower colour variation. Angrew. Chem. Int. Ed. Engl. 30: 17-33

Griesbach, R. J. and Klein, T. M. 1993. *In situ* genetic complementation of a flower mutant in *Doritis pulcherrima* (Orchidaceae). Lindleyana 8: 223-226.

Griesbach, R. J. 1994. An improved method for transforming plants through electrophoresis. Pl. Sci. 102: 81-89.

Grotewold, E. 2006. The genetics and biochemistry of floral pigments. Annu. Rev. Pl. Biol. 57: 761-780

Han, S. S. 1992. Role of sugars in bud development and vase life of cut *Liatris spicata*. Hort. Sci. 27: 1198-1200

Han, Y. Y., Ming, F., Wang, J. W., Wen, J. G., Ye, M. M. and Shen, D. L. 2006. Cloning and Characterization of a Novel Chalcone Synthase Gene from *Phalaenopsis hybrida* orchid flowers. Russian J. Pl. Physiol. 53: 223-230

Hanumappa, M., Choi, A., Ryu,S. and Choia, G. 2007. Modulation of flower colour by rationally designed dominant - negative chalcone synthase. J. Exp. Bot. 58: 2471-2478

Harborne, J. B. 1994. The flavonoids: Advances in Research Since 1986. Chapman and Hall, London, p. 265

Harborne, J. B. and Williams, C. 2000. Advances in flavonoid research since 1992. Phytochemistry 55: 481-504

Haun, R. S., Serventi, I. M. and Moss, J. 1992. Rapid, reliable ligation – independent cloning of PCR products using modified plasmid vectors. Bio Techniques 13: 515-518

Heiber. A. D., Mudalige-Jayawickrama, R.G. and Kuehnle, A. R. 2006. Colour genes in the orchid *Oncidium* Grower Ramsey: Identification, expression, and potential genetic identification in an interspecific cross. Plank 223: 521-531

Helariutta, Y., Elomaa, P., Kotilainen, M., Seppanen, P. and Teeri, T. H. 1993. Cloning of cDNA coding for dihydroflavonol 4-reductase (DFR) and characterization of *dfr* expressin in the corollas of *Gerbera hybrida* var. Regina (Compositae). Pl. Mol. Biol. 22: 183-193

Heller, W. and Forkmann, G. 1993. The Flavonoids. In: Harborne, J. B. (ed.). Advances in Research Since 1986, Chapman and Hall, London. p. 499-535

Hemsley, A., Arnheim, N., Toney, M. D., Cortopassi, G. and Galas, D. J. 1989. A simple method for site - directed mutagenesis using the polymerase chain reaction. Nucleic Acids Res. 17: 6545-6551

Higgins, D. G. and Sharp, P. M. 1988. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene 73: 237-244

Himi, E. and Noda, K. 2004. Isolation and location of three homologous dihydroflavonol 4-reductase (*dfr*) genes of wheat and their tissue - dependent expression. J. Exp. Bot. 55: 365-375

Hinnisdaeles, S., Del-Favero, J. and Vauterin, M. 1996. Direct cloning of PCR products amplified with two DNA polymerase. Bio Techniques 20: 186-188

Holton, T. A. and Graham, M. W. 1991. A simple and efficient method for direct cloning of PCR products using ddT-tailed vectors. Nucleic Acids Res. 19: 1156

Holton, T. A., Brugliera, F., Lester, D. R., Tanaka, Y., Hyland, C. D., Menting, J. G. T., Lu, C. Y., Francy, E., Stevenson, T. W. and Cornish, E. C. 1993. Cloning and

expression of cytochrome p450 genes controlling flower colour. Nature 366: 276-279.

Holton, T. A. and Cornish, E. C. 1995. Genetics and biochemistry of anthocyanin biosynthesis. Pl. Cell. 7: 1071–1083.

Honda, T. and Saito, N. 2002. Recent progress in the chemistry of polyacylated anthocyanins as flower colour pigment. Heterocycles 56: 633-692

Hornes, E. and Korsnes, L. 1990. Magnetic DNA hybridization properties of oligonucleotide probes attached to superparamagnetic beads and their use in the isolation of poly(A) mRNA from eukaryotic cells. Genet. Anal. Tech. Appl. 7: 145-150

Hosokawa, K., Fukunaga, Y., Fukushi, E. and Kawabata, J. 1996. Production of acylated anthocyanins by blue flowers of *Hyacinthus orientalis* regenerated *in vitro*. Phytochemistry 41: 1531-1533

Houwelingen, A., Souer, E., Spelt, K., Kloos, D., Mol, J. and Koes, R. 1998. Analysis of flower pigmentation.mutants generated by random transposon mutagenesis in *Petunia hybrida*. Pl. J. 13: 39-50

Hrazdina, G. 1988. Purification and properties of a UDP-glucose: flavonoid 3-O-glucosyltransferase from *Hippeastrum* petals. Biochem. Biophys. Acta. 955: 301-309

Hsieh, Y. H. and Huang, P. L. 1995. Studies on genetic transformation of *Phalaenopsis via* pollen tube pathway. J. Chinese Hort. Sci. 41(4): 309-324.

Hu, G. 1993. DNA polymerase - catalyzed addition of non templated extra nucleotides to the 3' end of a DNA fragment. DNA Cell Biol. 12: 763-770

Iandolino, A. B., da Silva, F. G., Lim. H., Choi, H., Williams, L. E. and Cook, D. R. 2004. High Quality RNA, cDNA, and derived EST libraries from grape vine (*Vitis vinifera* L.). Pl. Mol. Biol. Rep. 22: 269-278

Iida, S., Hoshino, A., Johzuka-Hisatomi, Y., Habu, Y. and Inagai, Y. 1999. Floricultural traits and transposable elements in the Japanese and common morning glories. Ann. N. Y. Acad. Sci. 870: 265-274

Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. 1990. PCR protocols: A Guide to Methods and Applications. Academic Press, San Diego, California, p.60-66

Jaakola, L. 2003. Flavonoid biosynthesis in bilberry (*Vaccinus myritillus* L.). M. Sc. thesis. University of Oulu, Finland, p. 124

Jacobsen, K. S., Breivold, E. and Horsnes, E. 1990. Purification of mRNA directly from crude plant tissues in 15 minutes using magnetic oligo dT microspheres. Nucleic Acids Res. 18: 3669

Jingwen, W., Feng, M., Yingying, H. and Daleng, S. 2006. Flavonoid 3'5'- hydroxylase from *Phalaenopsis*: A novel member of cytochrome p450, its cDNA cloning, endogenous expression and molecular modeling. Biotechnol. Lett. 28: 327-334(8)

Johan, S. N. O., Schneiders, H., Kool, A. J. And van Griesen, M. Q. J. M. 1995. Breeding of transgenic orange *Petunia hybrida* varieties. Euphytica 84:175-181.

Johnson, E. T., Yi, H., Shin, B., Oh, B. J, Cheong, H. and Choi, G. 1999. *Cymbidium hybrida* dihydroflavonol 4-reductase does not efficiently reduce dihydrokaemphero to produce orange pelargonidin – type anthocyanins. Pl. J. 19: 81-85.

Johnson, E. T., Ryu, S., Yi, H., Shin, B., Cheong, H. and Cho, G. 2001. Alteration of a single amino acid changes the substrate specificity of dihydroflavonol 4-reductase. Pl. J. 25(3): 325-333

Jones, D. H. and Howard, B. H. 1991. A rapid method for recombination and site-specific mutagenesis by placing homologous ends on DNA using polymerase chain reaction. Bio Techniques 10: 62-66

Kaluz, S., Kolbe, K. and Reid, K. B. 1992. Directional cloning of PCR products using exonuclease III. Nucleic Acids Res. 20: 4369-4370

Kaluz, S. and Flint, A. P. F. 1994. Ligation - independent cloning of PCR products with primers containing non base residues. Nucleic Acids Res. 22: 4845

Kanno, Y., Noda, N., Kazuma, K., Tsugawa, H. and Suzuki, M. 2003. Transformation of *Lobelia erinus*. In: The Abstract of 21st Annual Meeting of Jap. Soc. Plant Cell Mol. Biol. p. 121.

Kawabata, S., Ohta, S. M., Kusuhara, Y. and Sakiyama, R. 1995. Influence of low light intensities on the pigmentation of *Eustoma grandiflorum* flowers. Acta. Hort. 405: 173-178

Kay, Q. Q. N., Daoud, H. S. and Stirton, C. H. 1981. Pigment distribution, light reflection and cell structure in petals. Bot. J. Linn. Soc. 83: 57-84.

Koes, R., Quattrochio, F. and Mol, J. 1994. The flavonoid biosynthetic pathway in plants: function and evolution. BioEssays 16: 123-132

Kondo, T., Yoshida, K., Nakagawa, A., Kawal, T., Tamura, H. and Goto, T. 1992. Structural basis of blue - colour development in flower petals from *Commelina communis*. Nature 358: 515-518.

Kotewicz, M. L., Sampson, C. M., D' Alessio, J. M. and Gerard, G. F. 1988. Isolation of a cloned moloney murine leukemia virus reverse transcriptase lacking ribonuclease H activity. Nucleic Acids Res. 16: 265-277

Kreuz, K. R., Tommasini, E. and Martinoia. 1996. Old enzymes for a new job. Herbicide detoxification in plants. Pl. Physiol. 111: 349-353

Kreuzaler, F., Ragg, H., Fauz, E., Kuhn, D.N. and Hahlbrock, K. 1983. UV - induction of chalcone synthase mRNA in cell suspension cultures of *Petroselinum hortense*. Proc. Nat. Acad. Sci. USA. 80: 2591-2593

Kristiansen, K. N. and Rohde, W. 1991. Structure of the *Hordeum vulgare* gene encoding dihydroflavonol 4-reductase and molecular analysis of *antl8* mutants blocked in flavonoid synthesis. Mol. Gen. Genet. 230: 49-59

Kubasek, W. L., Ausubel, F. M. and Shirley, B. W. 1998. A light - independent developmental mechanism potentiates flavonoid gene expression in *Arabidopsis* seedlings. Pl. Mol. Biol. 37(2): 217-23

Kuehnle, A. R. and Sugii, N. 1992. Transformation of *Dendrobium* orchid using particle bombardment of protocorns. Pl. Cell Rep. 11: 484-488.

Kuehnle, A. R. 1997. Molecular biology of orchids. In: Arditti, J. and Pridgeon, A. M. (ed.). Orchid Biology: Reviews and Perspectives VII. Kluwer Acad. Publ. Dordrecht, Netherlands, p. 75-115.

Kuehnle, A. R., Lewis D. H., Markham, K. R., Mitchell, K. A., Davies, K. M. and Jordan, B. R. 1997b. Floral flavonoids and pH in *Dendrobium* orchid species and hybrids. Euphytica 95: 187-194.

Kuiper, D., van Reenen, H. S. and Ribot, S. A. 1991. Effect of gibberelic acid on sugar transport in to petals of 'Madelon' rose flowers during bud opening. Acta Hort. 298: 93-98

Kumar, A. and Linderberg, U. 1972. Characterization of messenger ribonucleoprotein and messenger RNA from KB cells. Proc. Nat. Acad. Sci. USA. 69: 681

Lekha Rani, C. 2002. Intra and Inter specific hybridization in *Dendrobium* spp. PhD. Thesis. Kerala Agicultural University, Thrissur, p. 360.

Liao, L. J., Pan, I. C., Chan, Y. L, Hsu, Y. H, and Chan, M. T. 2004. Transgene silencing in *Phalaenopsis* expressing the coat protein of *Cymbidium* Mosaic Virus is a manifestation of RNA- mediated resistance. Mol. Breeding. 13: 229-242.

Liau, C. H., Lu, J. C., Prasad,V., Hsiao, H. H, You, S. J., Lee, J. T, Yang, N. S., Huang, H. E, Feng, T. Y., Chen, W. H. and Chen, M. T. 2003a. The sweet pepper ferredoxin - like protein (pflp) conferred resistance against soft rot disease in *Oncidium* orchid. Transgenic Res. 12: 329-336

Liau, C. H., You, S. J., Prasad, V., Hsiao, H. H., Lu, J. C., Yang, N. S., and Chang, M. T. 2003b. *Agrobacterium* tumefaciens - mediated transformation of an *Oncidium* orchid. Pl. Cell Rep. 21: 993-998.

Linderberg, U. and Pesson, T. 1974. Messenger RNA isolation with poly (U) agarose. Methods Enzymol. 34: 496-499

Liu, D, Galli, M. and Crawford, N. M. 2001. Engineering variegated floral patterns in tobacco plants using the *Arabidopsis* transposable element Tag 1. Pl. Cell Physiol. 42: 419-423

Lo, C., Coolbaugh, R. C. and Nicholson, R. L. 2002. Molecular characterization and in silico expression of a chalcone synthase gene family in *Sorghum bicolor*. Physiol. Mol. Pl. Path. 61: 179-188

Logemann, J., Schell, J. and Willmitzer, L. 1987. Improved method for the isolation of RNA from plant tissues. Anal. Biochem. 163: 16-20

Loomis, W. D. 1974. Overcoming problems of phenolic and quinines in the isolation of plant enzyme and organelles. Methods Enzymol. 31: 528-548

Malnoy. M., Reynoird, J. P., Mourgues, F., Chevreau, E. and Simoneau, P. 2001. A method for isolating total RNA from pear leaves. Pl. Mol. Biol. Rep. 19: 69

Marchuk, D., Drumm, M., Saulino, A. and Collins, F. S. 1991. Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acids Res. 19:1154

Martin, C., Carpenter, R., Sommer, H., Saedler, H. and Coen, E. S. 1985. Molecular analysis of instability in flower pigmentation of *Antirrhinum majus*, following isolation of the Pallida locus by transposon tagging. EMSO J. 4: 1625-1630.

Maxam, A. M. and Gilbert, W. 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA, 74: 560-564

McIntosh, C. A. and Mansell, R. L. 1990. Biosynthesis of naringenin in Citrus paradisi: UDP - glucosyltransferase activity in grape fruit seedlings. Phytochemistry 29: 1533-38

Mead, D. A., Pey, N. K., Herrnstadt, C., Marcil, R. A. and Smith, L. M. 1991. A universal method for the direct cloning of PCR amplified nucleic acid. Bio Technology 9: 657-663

Meisel, L., Fonseca, B., Gonzalez, S., Baezayates, R., Cambiazo, V. and Silva, H. 2005. A rapid and efficient method for purifying high quality, total RNA from peaches (*Prunus persica*) for functional genomics analysis. Biol. Res. 38: 83-88

Mercy, S. T. and Dale, B. 1997. Orchids. St. Joseph press TVM, India, p. 132

Meyer, P., Heidemann, I., Forkmann, G. and Saedler, H. 1987. A new Petunia flower colour generated by transformation of a mutant with a maize gene. Nature 330: 677-678.

Miki, D., Itoh, R. and Shimamoto, K. 2005. RNAi silencing of single and multiple members in a gene family of rice. Pl. physiol. 138: 1903-1913

Mizutani, M., Tsuda, S., Suzuki, K., Nakamura, N., Fukui, Y., Kusumi, T. and Tanaka, Y. 2003. Evaluation of post - transcriptional gene silencing methods using flower colour as the indicator. Pl. Cell Physiol. 44: 122

Mol, J., Van der Krol A.R., Van Tunen, A. J.,Van Blokand, R., de Lange, P. and Stuitje, A. R. 1990. Regulation of plant gene expression by antisense RNA. FEBS lett. 268: 427-430

Mol, J., Jenkins, G., Schafer, E. and Weiss, D. 1996. Signal perception, transduction and gene expression involved in anthocyanin biosynthesis. Crit. Rev. Pl. Sci. 15: 525-557.

Mol, J., Grotewald, E. and Koes, R. 1998. How genes paint flowers and seeds? Trends Pl. Sci. 3: 212-217.

Mori, S., Kobayashi, H., Hoshi, Y., Kondo, M. and Nakano, M. 2004. Heterologous expression of the Flavonoid 3', 5'-hydroxylase gene of *Vinca major* alters flower colour in transgenic *Petunia hybrida*. Pl. Cell. Rep. 22: 415-421

Moscovici, S., Moalem-Beno, D. and Weiss, D. 1996. Leaf - mediated light responses in petunia flowers. Pl. Physiol. 110:1275-1282

Moyano, E., Portero-Robles, I., Medina-Esco-bar, N., Valpuesta, V., Munoz-Blanco, J. and Caballero, J. L. 1998. A fruit specific putative dihydroflavonol 4-reductase gene is differentially expressed in strawberry during the ripening process. Pl. Physiol. 117: 711-716

Mudalige-Jayawickrama, R. G., Champagne, M. M., Heiber, A. D., and Kuehnle, A. R. 2005. Cloning and characterization of two anthocyanin biosynthetic genes from *Dendrobium* orchid. J. Amer. Soc. Hort. Sci. 130: 611-618.

Mudalige, R. M. and Kuehnle, A. R. 2004. Orchid biotechnology in production and improvement. Hort. Sci. 39: 11–17

Murray, J. R. and Hackett, W. P. 1991. Dihydroflavonol activity in relation to differential anthocyanin accumulates in juvenile and mature phase. *Hedera helix* L. Pl. Physiol. 97: 343-351

Nakamura, N., Fukuchi-Mizutani, M., Suzuki, K., Miyazaki, K. and Tanaka, Y. 2006. RNAi suppression of the anthocyanidin synthase gene in *Torenia hybrida* yields white flowers with higher frequency and better stability than antisense and sense suppression. Pl. Biotechnol. 23: 13-18

Nakatsuka, T., Abe, Y., Kakizaki, Y., Yamamura, S. and Nishihara, M. 2007. Production of red - flowered plants by genetic engineering of multiple flavonoid biosynthetic genes. Pl. Cell Rep. 26: 1951-1959

Nan, G. L. and Kuehnle, A. R. 1995. Factors affecting gene delivery by particle bombardment of *Dendrobium* orchids. *In Vitro* Cell. Dev. Biol. 31: 131-136.

Nielsen, K.M. and Podivinsky, E. 1997. cDNA cloning and endogenous expression of a flavonoid 3', 5' - hydroxylase from petals of lisianthus (*Eustoma grandiflorum*). Pl. Sci 129: 167–174

Nielsen, K. M., Deroles, S. C., Markham, K. R., Bradley, J. M., Podivinsky, E. and Manson, D. 2001. Antisense flavonol synthase alters copigmentation and flower colour in lisianthus. Mol. Breed. 9: 217-229

Nishihara, M., Nakatsuka, T. and Yamamura, S. 2005. Flavonoid components and flower colour change in transgenic tobacco plants by suppression of chalcone isomerase gene. FEBS Lett. 579: 6074-6078

Nishihara, M., Nakatsuka, T., Hosokawa, K., Yokoi, T., Abe,Y., Mishiba, K. and Yamamura, S. 2006. Dominant inheritance of white - flowered and herbicide-resistant traits in transgenic gentian plants. Pl. Biotechnol. 23: 25-31

Nisson, P. E., Rashtchian, A. and Watkins, P. C. 1991. Rapid and efficient cloning of Alu - PCR products using uracil glycolyase. PCR Methods Appl. 1: 120-123

Noda, K., Glower, B. J., Linstead, P. and Martin, C. 1994. Flower colour intensity depends on specialized cell shape controlled by a Myb-related transcription factor. Nature 369: 661-664.

Nowak, B. H. 2008. Changes in anthocyanin content as indicator of maize sensitivity to selenium. J. Pl. Nutr. 31: 1232-1242

O'Driscoll, L., Daly, C., Saleh, M. and Clyness, M. 1993. The use of reverse transcriptase - polymerase chain reaction (RT-PCR) to investigate specific gene expression in multi drug resistant cells. Cytotechnology 12: 289-314

Pawlowski, K., Kunze, R., de Vries, S. and Bisseling, T. 1994. Isolation of total, poly(A) and polysomal RNA from plant tissues. In: Gelvin, S. B. and Schilperoof, R. A. (ed.). Plant Molecular Biology Manual. Kluwer Academic Publishers, Holland, p. 1-3

Phoka, N., Tragoonung, S. and Vanavichit, A. 2002. Anthocyanin intensity in rice grain is regulated by splicing efficiency of dihydroflavonol 4-reductase and is temperature sensitive. http://www. dna.kps.ku.ac.th

Poulton, J. E. 1990. Cyanogenesis in plants. Pl. Physiol. 94: 401-405

Prober, J. M., Trainor, G. L., Dam, R. J., Hobbs, F. W., Robertson, C. W., Zagursky, R.J., Cocuzza, A. J., Jensen, M. A. and Baumeister, K. 1987. A system for rapid DNA sequencing with fluorescent chain - terminating dideoxynucleotides. Science 238: 336-341

Que, Q., Wang, H. Y., English, J. J. and Jorgensen, R. A. 1997. The frequency and the degree of co - suppression by sense chalcone synthase transgene are dependent on transgene promoter strength and are reduced by premature nonsense codon in the transgene coding sequence. Pl. Cell 9: 1357-1368

Rastogi, S. C., Mendiratte, N. and Rastogi, P. Bioinformatics methods and applications. Genomics proteomics and Drug discovery. Prentice Hall of India Pvt. Ltd. New Delhi.

Reddy, A. R., Scheffler, B., Madhuri, G., Srivastava, M. N., Kumar, A., Sathyanarayanan, P. V., Nair, S. and Mohan, M. 1996. Chalcone synthase in rice (*Oryza sativa* L.): Detection of the CHS protein in seedlings and molecular mapping of the *chs* locus. Pl. Mol. Biol. 32: 735-743

Reif, H.J., Niesbach, U., Deumling, B. and Saedler, H. 1985. Cloning and analysis of two genes for chalcone synthase from *Petunia hybrida*. Mol. Gene. Genet. MGG 199: 208-215.

Robards, K. and Antolovich, M. 1997. Analytical chemistry of fruit bioflavonoids. Analyst. 122: 11-34

Rosati, C., Cadic A., Duron, M., Renoce, J. P. and Simoneau, P. 1997. Molecular cloning and expression analysis of dihydroflavonol 4-reductase gene in flower - organs of *Forsythia intermedia*. Pl. Mol. Biol. 35: 303-311.

Rosati, C., Simoneau, P., Treutter, D., Poupard, P., Cadot, Y., Cadic, A. and Duron, M. 2003. Engineering of flower colour in forsythia by expression of two independently - transformed dihydroflavonol 4-reductase and anthocyanidin synthase genes of flavonoid pathway. Mol. Breed. 12: 197-208

Rozen, S. and Skaletsky, H. J. 2000. Primer 3 on WWW for general users and for biologist programmers. In: Krawetz, S. and Misener, R. (ed.). Bioinformatics methods and Protocols; Methods in Molecular Biology. Humana press, Totowa, New Jersy, p. 110

Sachray, L. S., Weiss, D., Reuveni, M., Levi, A. N. and Shamir, M. O. 2002. Increased anthocyanin accumulation in aster flowers at elevated temperatures due to magnesium treatment. Physiol. Plant. 114: 559-565

Salzman, R. A., Fujita, T., Zhu-Salzman, K., Hasegawa, P. M. and Bressan, R. A. 1999. An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates. Pl. Mol. Biol. Rep. 17: 11-17

Sambrook, J. and Russel, D. W. 2001. Molecular cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, New York, p.18125

Sanger, F., Nicklen, S. and Coulson, A. R. 1977. DNA sequencing with chain terminating inhibitors. Proc. Nalt. Acad. Sci. USA, 74: 5463-5467

Scharf, S. J., Horn, G. T. and Erlich, H. A. 1986. Directional cloning and sequence analysis of enzymatically amplified genomic sequence. Science 233: 1076-1078

Schneitz, K. 2000. Hot phenol method for RNA isolation. http://www.unizh.ch/botinst/Cyto_Website/schneitzLab/Methods/ProtocolsRNA/hotPhenol.html

Schuldiner, A. R. and Tanner, K. 1997. Rapid (ligase-free) subcloning of PCR products. Methods Mol. Biol. 67: 69-78

Sela, M., Anfinsen, C. B. and Harington, W. F. 1957. The correlation of ribonuclease activity with specific aspects of tertiary structure. Biochem. Biophys. Acta 26: 502

Semiarti, E., Indrianto, A., Purwantoro, A., Isminingsih, S., Suseno, N., Ishikawa, T., Yoshioka, Y., Machida, Y. and Machida, C. 2007. *Agrobacterium* – mediated transformation of the wild orchid species *Phalaenopsis amabilis*. Pl. Biotechnol. 24: 265-272

Shimada, Y., Nakano-Shimada, R., Obhayashi, M., Okinaka, Y., Kiyokawa, S. and Kikuchi, Y. 1999. Expression of chemeric p450 genes encoding –flavonoid -3', 5' -hydroxylase in transgenic tobacco and petunia plants. Met. Eng. 4: 67-79.

Sibhatu, M. B. 2003. Cloning and sequencing of Glucosyltransferase (GT) Genes from Grapefruit (*Citrus paradisi*) Seedlings: Searching for 7-O-GT. M. Sc. thesis. East Tennesse State University, p. 164

Singh, F. 1986. Orchids. In: Chandha, K. L. and Chowdhary, B. (ed.). Ornamental Horticulture in India. Indian Counsil of Agricultural Research, New Delhi, p.127-153

Smith, C., Day, P. J. and Walker, M. R. 1993. Generation of Cohesive ends on PCR products by UDG - mediated extension of dU, and application for cloning in to restriction digest - linearized vectors. PCR Methods Appl. 2:328-332

Smith, L. M., Sanders, J. Z., Kaiser, R. J., Hughes, P., Dodd, C., Connell, C. R., Heiner, C., Kent, S. B. and Hood, L. E. 1986. Fluorescence detection in automated DNA sequence analysis. Nature 321: 674-678

Solfanelli, C., Alessandra, P., Loreti, E., Alpi, A. and Perata, P. 2006. Sucrose - specific induction of anthocyanin biosynthetic pathway in *Arabidopsis*. Pl. Physiol. 140(2): 637-646

Sparvoli, F., Martin, C., Scienza, A., Gavazzi, G. and Tonelli, C. 1994. Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grapes (*Vitis vinifera* L.). Pl. Mol. Biol. 24: 743-755

Stafford, H. A. 1990. Flavonoid metabolism. CSR Press. Boca Raton

Strack,D. and Wray, V. 1993. The Anthocyanins. In: J. B. Harbourne (ed.). The Flavonoids: Advances in Research since 1986. Chapman and Hall, London, UK. p 1-22

Su, V. and Hsu, B. D. 2003. Cloning and expression of putative cytochrome p450 gene that influence the colour of *Phalaenopsis* flowers. Biotechnol. Lett. 25: 1933-1939.

Szerszen, K., Szcyglowski, R. S. and Bandurski. 1994. *iaglu*, a gene from *Zea mays* involved in conjugation of growth hormone indole-3-acetic acid. Science 265: 1699-1701

Tanaka, Y. 2006. Flower colour and cytochrome P450. Phytochem. Rev. 5: 283-291

Tanaka, Y., Yonekura, K., Fukuchi-Mizutani, M., Fukui, Y., Fujiwara, H., Ashikari, T. and Kusumi, T. 1996. Molecular and biochemical characterization of three anthocyanin synthetic enzymes from *Gentiana triflora*. Pl. Cell Physiol. 37: 711–716

Tanaka, Y., Tsuda, S. and Kusumi, T. 1998. Metabolic engineering to modify flower colour. Pl. Cell. Physiol. 39: 1119-1126.

Tanaka, Y., Katsumoto, Y., Brugliera, F. and Jonh, M. 2005. Genetic engineering in floriculture. Pl. Cell. Tissue Org. Cult. 80: 1-24

Tanaka, Y. and Brugliera, F. 2006. Flower colour. In: Ainsworth, C. (ed.). Flowering and its manipulation. Black-well, London, p. 201- 239

Thomson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. 1997. The Clustal X windows interference: fexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24: 4876-4882

Toguri, T., Umemoto, N., Kobayashi, O. and Ohtani, T. 1993. Activation of anthocyanin synthesis gene by white light in egg plant hypocotyl tissues, and identification of an inducible P-450 cDNA. Pl. Mol. Biol. 23: 933-946

Tsuda, S., Fukui, Y., Nakamura, N., Katsumoto, Y., Yonekura- Sakakibara, K., Fukuchi-Mizutani, M., Ohira, K., Ueyama, Y., Ohkawa, H., Holton, T. A., Kusumi, T. and Tanaka, Y. 2004. Flower colour modification of *Petunia hybrida* commercial varieties by metabolic engineering. Pl. Biotechnol. 21: 377-386

Tsukaya, H., Ohshima, T., Naito, S., Chino, M. and Komeda, Y. 1991. Sugar - dependent expression of the *CHS-A* gene for chalcone synthase from Petunia in transgenic *Arabidopsis*. Pl. Physiol. 97: 1414-1421

Ueyama, Y., Suzuki, K., Fukuchi-Mizutani, M., Fukui, Y., Miyazaki, K., Ohkawara, H., Kusumi, T. and Tanaka, Y. 2002. Molecular and biochemical characterization of torenia flavonoid 3' hydroxylase and flavone synthase II and modification of flower colour by modulating the expression of these genes. Pl. Sci.163: 253-263

Ueyama, Y., Katsumoto, Y., Fukui, Y., Fukuchi-Mizutani, M., Ohkawa, H., Kusumi, T., Iwashita, T. and Tanaka, Y. 2006. Molecular characterization of the flavonoid biosynthetic pathway and flower colour modification of *Nierembergia* sp. Pl. Biotenol. 23: 19-24

Van Blokland, R., Van der Geet, N., Mol, J. N. M., and Kooter, J. 1994. Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. Pl. J. 6: 861-867

Venkatachalam, P., Thanseem, I. and Thulaseedharan, A. 1999. A rapid and efficient method for isolation of RNA from bark tissues of *Hevea brasiliensis*. Curr. Sci. 77: 635-637

Wang, C. S. and Vodkin, L. O. 1994. Extraction of RNA from tissues containing high levels of procyanidins that bind RNA. Pl. Mol. Biol. Rep. 12: 132-145

Wang, M. B. and Waterhouse, P. M. 2001. Application of gene silencing in plants. Curr. Opin. Pl. Biol. 5:146-150

Wang, T., Zhang, N. and Du, L. 2005. Isolation of RNA of high quality and yield from *Ginkgo biloba* leaves. Biotechnol. Lett. 27: 629-633

Weiss. D. 2000. Regulation of flower pigmentation and growth: multiple signaling pathways control anthocyanin synthesis in expanding petals. Physiol. Planta. 110: 152-157

Wha-Shin Hsu, Yi-Yin, D. and Pung-Ling, H. 1997. Isolation and characterization of a cDNA Encoding Chalcone Synthase Homolog from *Phalaenopsis*. Pl. Physiol. 113:1465

Winkel-Shirley, B. 2001. Flavonoid biosynthesis. A colourful model for genetics, biochemistry, cell biology and biotechnology. Pl. Physiol. 126: 485-493

Wreschner, D. H. and Herzberg, M. 1984. A new blotting medium for the simple isolation and identification of highly resolved messenger RNAs. Nucleic Acids Res.12: 1349-1359

Yamaguchi, T., Fukuda-Tanaka, S., Inagaki Y., Saito N., Yonekura-Sakakibara, K., Tanaka Y., Kusumi T. and Iida, S. 2001. Genes encoding the vacuolar Na+/H+ exchanger and flower colouration. Pl. Cell Physiol. 42: 451-461

Yamane, T., Jeong, S., Yamamoto, N.G., Koshita, Y. and Kobayashi, S. 2006. Effect of temperature on anthocyanin biosynthesis in grape berry skins. In: Am. J. Enol. Vit. 57(1): 54-59.

Yang, J., Lee, H. J., Shin, D. H., Oh, S. K., Seon, J. H., Paek, K. Y. and Han, K. H. 1999. Genetic transformation of *Cymbidium* orchid by particle bombardment. Pl. Cell Rep. 18: 978-984

Yoshida, K., Kondo, T., Okazaki, Y. and Katou, K. 1995. Cause of blue petal colour. Nature 373: 291

Yu, H., Yang, S. H. and Goh, C. J. 2001. *Agrobacterium* mediated transformation of *Dendrobium* orchid with class 1 knox gene DOH1. Pl. Cell. Rep. 20: 301-305

Yu, O., Matsuno, M. and Subramanian, S. 2006. Flavonoid Compounds in Flowers: Genetics and Biochemistry. Flor. Orna. Pl. Biotechnol. 1: 82-92

Zeng, Y. and Yang, T. 2002. RNA isolation from highly viscous samples rich in polyphenols and polysaccharides. Pl. Mol. Biol. Rep. 20: 417

Zhong, J. J., Seki, T., Kinoshita, S. I. and Yoshida, T. 1991. Effect of light irradiation on anthocyanin production by suspended culture of *Perilla frutescens*. Biotechnol. bioeng. 38: 653-658

Zhuang, C.X., Yau, C.P. and Zee, S.Y. 1999. Differential expression of a putative dihydroflavonol reductase gene in rice (Accession No. AF 134807). Pl. Physiol. 120: 633

Appendices

APPENDIX I

1X TAE buffer composition

Tris Acetate	0.04 M
EDTA	0.001M
pH 8.0	

APPENDIX II

S. O. C. medium components

Tryptone	2.0%
Yeast extract	0.5%
NaCl	10mM
KCL	25mM
$MgCl_2$	10mM
$MgSo_4$	10mM
Glucose	20mM
pH	7.0

Store at RT or +4° C

APPENDIX III

Reagents for plasmid DNA isolation

1. Solution I

Glucose	50 mM
Tris-Cl	25 mM (pH 8.0)
EDTA	10 mM (pH 8.0)

2. Solution II

NaOH	0.2 N (Freshly diluted from 10N stock)
SDS	1.0 %

3. Solution III

Pottasium Acetate (5M)	60.0 ml
Glacial Acetic acid	11.5 ml
Distilled water	29.5 ml
Total volume	100 ml

4. L.B medium (pH 7.0)

Yeast extract	5.0 g
Tryptone	10.0 g
NaCl	10 g
Distilled water	1000 ml

ISOLATION AND CHARACTERIZATION OF cDNA ENCODING DIHYDROFLAVONOL 4-REDUCTASE GENE FROM ORCHID *DENDROBIUM* VARIETY SONIA17.

SARITHA, V. S.

Abstract of the
thesis submitted in partial fulfilment of the requirement
for the degree of

Master of Science in Agriculture

Faculty of Agriculture
Kerala Agricultural University, Thrissur

2009

Department of Plant Biotechnology
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM 695522

Abstract

ABSTRACT

The thesis entitled "Isolation and characterization of cDNA encoding dihydroflavonol 4-reductase gene from *Dendrobium* orchid variety Sonia 17" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2006-2008 with an objective of characterizing one of the key genes involved in anthocyanin biosynthesis, viz., dihydroflavonol 4-reductase from flower buds of *Dendrobium* Sonia 17 orchid.

Reverse Transcription-PCR technique was used in the present study for the amplification of cDNA encoding *dfr* gene. Heterologous forward and reverse primers were designed based on the gene sequences of *Cymbidium* orchid and *Oryza sativa* using Primer 3 software. Total RNA isolated from the flower buds using TRIZOL reagent yielded good quality intact RNA, with no genomic DNA contamination and showed an A_{260}/A_{280} ratio of 1.6-2.0. The cDNA synthesized from purified mRNA was amplified using gene specific primers designed. All the primers included in the study yielded reproducible amplification. The primer sets A (Af and Ar) and C (Cf and Cr) which were designed based on the *dfr* gene sequence of *Cymbidium* orchid yielded amplified fragments of size approximately 400-500 bp and 100 bp respectively. Primer set B (Bf and Br) synthesized from *Oryza sativa dfr* gene sequences yielded an amplified product of size nearly 300 bp.

The cDNA amplified with primer set A was cloned in pGEM-T Easy Vector and multiplied in competent *E. Coli* strain DH5α for sequencing. The transformed *E. Coli* colonies were grown on L.B agar plates and selected by blue – white screening. Three white colonies were randomly selected and plasmid DNA was isolated from transformed clones. The presence of insert was checked by PCR analysis of the plasmid DNA with gene specific primer (primer set A). The agarose gel electrophoresis showed the presence

of amplified fragment. Sequencing of two clones at Chromous Biotech Pvt. Ltd. using T7 and SP6 primer failed to amplify the plasmid DNA.

This work was an initial step towards isolation and characterization of dihydroflavonol 4-reductase gene in *Dendrobium* variety Sonia 17. The primers designed in this study could successfully amplify the cDNA encoding *dfr*. PCR reactions gave good results but the sequencing was not satisfactory. For the better understanding of the *dfr* sequences, the sequencing reaction needs to be repeated with other clones.