ISOLATION AND CHARACTERIZATION OF cDNA ENCODING CHALCONE SYNTHASE FROM FLOWER BUDS OF ORCHID *Dendrobium* VARIETY SONIA 17.

ANJANA, G.R

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Department of Plant Biotechnology
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM 695522

CERTIFICATE

Certified that this thesis entitled **"Isolation and Characterization of Chalcone Synthase from flower buds of Orchid, *Dendrobium* variety Sonia 17"** is a record of research work done independently by Mrs. Anjana, G.R (2004-11-34) 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
17/12/2007

Dr. K. B. SONI
(Major Advisor, Advisory Committee)
Associate Professor
Department of Plant Biotechnology,
College of Agriculture, Vellayani,
Thiruvananthapuram – 695 522

APPROVED BY

Major Advisor:

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

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

Dr. K. UMAMAHESWARAN
Associate Professor
Department of Plant Pathology,
College of Agriculture, Vellayani,
Thiruvananthapuram – 695 522

EXTERNAL EXAMINER

Dr. K. NIRMAL BABU
Principal Scientist
Crop Improvement and Biotechnology
Indian Institute of Spices Research
Calicut – 673 012

DECLARATION

I hereby declare that this thesis entitled "**Isolation and characterisation of cDNA encoding chalcone synthase from flower buds of orchid *Dendrobium* variety Sonia 17**" is a bonofide 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
17/12/2007

ANJANA, G.R
(2004- 11-34)

Dedicated to
My Beloved Parents
and Husband

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LIST OF ABBREVIATIONS

A	adenine
Acc. No.	Accession number
AMV	Avian myeloblastosis virus
ANS	Anthocyanin synthase
BME	β- Mercaptoethanol
BLAST	Basic Local Alignment Search Tool
bp	base pair
C	cytosine
CHS	Chalcone synthase
cM	centi Morgan
CTAB	cetyl trimethyl ammonium bromide
cDNA	Complementary DNA
CoA	Co enzyme A
DEPC	Diethyl pyrocarbonate
DFR	Dihydroflavonol 4- reductase
DNA	deoxy ribonucleic acid
dNTP	deoxynucleotide triphosphate
d(T)	deoxy thymydilic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
FASTA	Fast All
G	guanine
GI	gene identity number
LiCl	Lithium Chloride
M	Molar
mM	millimolar
MMuLV	Moloney Murine Leukemia virus
mRNA	Messenger RNA
NCBI	National Centre for Biotechnology Information

ng	nanogram
PCR	Polymerase Chain Reaction
poly(A)	poly adenosine
PVP	Polyvinyl pyrrolidone
RACE	Rapid amplification of cDNA ends
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	Reverse Transcription- PCR
SDH	Shikmate dehydrogenase
T	thymine
TAE	Tris acetate EDTA
Taq	Thermus aquaticus
TE	Tris-HCl –EDTA
Tris	Tris (hydroxy methyl) aminomethane
Tris-HCl	Tris (hydroxy methyl) aminomethane hydrochloride
U	uracil
UFGT	UDP: flavonoid 3-Oxy- glucosyl transferase
µl	microlitre
µg	microgram

1. Introduction

1. INTRODUCTION

Orchids are one of the most beautiful flowers which represent a highly evolved family among monocotyledons with 600-800 genera and 25,000-35,000 species (Singh, 1986). They exhibit an incredible range of diversity in size, shape and colour of their flowers. Orchids occupy a prime position among all the flowering plants valued for cut flower production and as potted plants. They are known for their long lasting and beautiful flowers that fetch high price in the market. The orchid cut flower industry is growing at an annual rate of 10-20 per cent (Pradhan, 2001) and their ornamental value account for multimillion dollars. Among the commercial orchids, sympodial types rank higher in export market. Of these, the genus *Dendrobium* is currently enjoying very high popularity in both domestic (Lekha Rani, 2002) and international markets. It is the second largest genera in the orchid family with more than 1000 species. *Dendrobium* hybrids are the most suitable and popular among the commercial orchids grown in Kerala. Sonia (*D. caesae* x *D. Tomie Drake*) is well known for its attractive flowers.

Orchid flowers are striking for their specific patterns of colors in sepals, petals, and the modified dorsal petals (lips). Dendrobium flowers have enormous variation in flower colour ranging from white, mauve, pink, red, blue, purple to yellow. Flower spikes are medium sized with flowers ranging from 5 to 20 numbers (Mercy and Dale, 1997). Flower colour is a qualitative trait. New varieties remain at the prime position only for a short period. The breeders are attempting to introduce novel varieties with distinct traits. Traditional breeding has its own limitations as it is time consuming and laborious. Genetic engineering offers an option for the incorporation of desirable genetic traits in a faster and more precise manner complimenting traditional breeding. Genetic transformation is being effectively used for the rapid improvement of floricultural crops world over.

Market value of orchid flowers is determined by different factors like flower colour, pattern of colour, length of spike and number of flowers in the spike. Among

these flower colour is an important factor. Flower colour is influenced by combination of factors like the type of pigments present in the flower, the translocation of such pigments from the site of production and the pH of the cell vacuoles. Anthocyanin is the major pigment responsible for flower colour in orchid and is regulated by different genes, most of which encode enzymes relevant to the flavonoid pathway. These genes include chalcone synthase, dihydroflavonol 4-reductase and flavanone 3-hydroxylase. However, molecular and genetic studies of these genes are too limited to figure out the fundamental factors regulating the floral coloration of orchids, although the transgenic approach has been adopted for the study of *Cymbidium hybrida* DFR in a heterologous petunia system. The regulation of pigmentation is refined to specific cells of the different floral organs, and the expression of genes involved in flavonoid synthesis may be the initial steps in the complex regulation of pigmentation.

To understand the development of color patterns in flowers, it will be also important to identify additional regulatory genes in the flavonoid pathway. Further investigations of their regulatory mechanisms and pathways will provide strategies toward genetic engineering of color in orchid flower, which is now possible with efficient and reliable transformation systems. Genetic modification of the flavonoid pathway has successfully added novel flower color varieties in those cases like, violet/blue carnation and roses in which efficient transformation systems for the target species and molecular tools for flower colour modification are available. Since now only very few reports are available regarding the gene expression and regulation in *Dendrobium* orchid. Till date, no report has been published in India regarding gene regulation in *Dendrobium* orchid.

In view of the above facts, present investigation was conducted with an objective of isolation and characterization of cDNA of chalcone synthase from immature flower buds of orchid, *Dendrobium* variety Sonia 17 which could ultimately be utilized for studying the regulation of gene expression.

2. Review of Literature

2. REVIEW OF LITERATURE

2.1 Introduction

Orchids are very distinctive ornamentals. They occupy 13 per cent of the total land area devoted to cut flower (Singh, 2005). Orchid growing is a multi-million industry, most notably in the cut flower trade (Khairol and Auni, 1991). *Dendrobiums* are the popular commercial orchid grown in Kerala. Approximately 24.3 million stalks of orchid cut-flowers were produced in 2000 with *Dendrobium* topping the list at 13.1 million stalks. They are mass-propagated by the floral industry and widely commercialized for their decorative value.

Flower colour is the most important factor, which decides the market value of orchid flowers. Dendrobium flowers are usually in colors such as white, mauve, pink, red, blue, purple and yellow (Mercy and Dale, 1997). Improved novel flowered varieties will definitely fetch high price in the market, as the consumers prefer the same.

Flower color originates primarily from flavonoids and their colored class compounds, anthocyanins (Harborne and Williams, 2000). In addition to the anthocyanin structures, co-existing co-pigments such as flavonols and flavones, metal ions and vacuolar pH give infinite array of flower colors from orange to blue (Harborne and Williams, 2000; Tanaka et al., 2005). The flavonoid biosynthetic pathway leading to aglycons is well established and conserved among plant species and most structural genes leading to their synthesis have been obtained (Tanaka et al., 2005).

Hybridization breeding has contributed to increasing the variety of available colors. However, it is still uncommon for a plant species to have a wide variety of colors because the factors influencing flower color are, as a rule, genetically regulated.

It is difficult to increase the variety of flower color by using hybridization breeding alone because of the limited gene source within a species (Tanaka et al., 2005). For example, carnations and roses do not have violet/blue varieties due to lack of the flavonoid 3,5-hydroxylase (*F'3, 5'H*) gene, which is necessary to synthesize delphinidin (Holton et al., 1993). Petunia and tobacco do not accumulate pelargonidin due to the substrate specificity of their dihydroflavonol 4-reductase (DFR); as a result, they lack brick-red varieties (Forkmann and Ruhnau, 1987).

On the other hand, genetic engineering of the flavonoid pathway has successfully added novel flower color varieties in those cases in which efficient transformation systems for the target species and molecular tools for flower colour modification are available (Tanaka et al., 2005; Forkmann and Martens, 2001). Violet/blue carnations and roses (Holton, 1995; Tanaka et al., 2005) and brick-red petunias (Meyer et al., 1987; Helariutta et al., 1993; Tanaka et al., 1995) have been obtained, and such carnations are on the market. Reliable regeneration protocol and efficient protocol for genetic transformation have already been standardized for the *Dendrobium* variety Sonia 17 (Swarnapiria, 2004).

2.2 Flavonoids in Plants

The ability to synthesise numerous natural products or secondary metabolites including phenolics, alkaloids and terpenoids is the unique biochemical characteristic of higher plants. These diverse plant natural products are generally organized in terms of their biosynthetic patterns and their chemical compositions. Out of all the secondary metabolites, phenolics are known to constitute the majority of plant based aromatic natural products that exist in a number of categories such as simple phenols, phenyl propanoids, flavonoids, tannins and quinones (Kaufman et al., 1999).

2.3 The Chemical Structure of Flavonoids

Flavonoids are a class of low-molecular-weight phenolic compounds that are widely distributed in the plant kingdom. Over 6000 naturally occurring flavonoids have been described, and many of them are common in higher plants (Harborne and Williams, 2000). These compounds frequently serve as pigments in plants, but are also involved in many biological interactions. Flavonoids are built upon a C6-C3-C6 flavone skeleton in which the three-carbon bridge between the phenyl groups is commonly cyclized with oxygen. Based on the degree of unsaturation and oxidation of the three-carbon segment, flavonoids are divided in to several classes. Most flavonoids reported in the literature are glycosides of a relatively small number of flavonoid aglycons, which are generally water-soluble and accumulating in the vacuoles of plant cells (Bohm, 1998; Seigler, 1998).

2.4 Role of Flavonoids in Plants

Flavonoids are water soluble pigments produced by all higher plants. These products were once considered to be waste metabolites that didn't participate in plant primary activities like growth, development and reproduction. Today, roles of flavonoids in the life cycle of plants are becoming increasingly apparent (Brouillard and Dangels, 1993; Stafford, 1991). Anthocyanins, perhaps the most widely known class of flavonoids, are responsible for the blue (delphinidins), red (anthocyanins) and purple (pelargonidins) coloration to fruits, vegetables and flowers in higher plants (Martin and Gerats, 1993). Anthocyanins in fruits and flowers serve as visual signals that attract insects and animals, thus playing an important role in the ecology of pollination and seed dispersal (Holton and Cornish, 1995; Shirley, 1996; Mol et al., 1998). Some flavonoids act as signal molecules in the interaction between legumes and nodulating bacteria, and many can modulate plant insect interactions by acting as feeding deterrents or attractants. Some are known for their contribution of sweet or bitter tastes

in food. Of all plant secondary compounds, anthocyanins have been investigated most extensively in the areas of chemistry, biochemistry and genetics (Jaakola, 2003).

A significant role of flavonoids that has been under very active research is their possible beneficial influence on human health. Flavonoids have been found to own potent antioxidant and free radical scavenging activities *in vitro*. There is growing evidence from human consumption studies supporting a protective role of flavonoids in cardiovascular diseases and cancer. Many flavonoids have been found to possess antiviral, antibacterial, antifungal or anti-allergic properties. However, because of the wide variety of different flavonoids, their possible interactions with other substances, and the complexity of their metabolism in the human system, more research in this area is still needed (Hertog et al., 1995; Hollman et al., 1996; Peterson and Dwyer, 1998; Ross and Kasum, 2002).

2.5 Anthocyanin Biosynthesis Pathway

The anthocyanin biosynthetic pathway has been well established and many genes encoding anthocyanin biosynthetic enzymes have been characterized and cloned (Dooner et al., 1991). Flavonoids are synthesized *via* the phenyl propanoid pathway. Two classes of genes are required for anthocyanin biosynthesis, the structural genes encoding enzymes that directly participate in the formation of anthocyanins and other flavonoids, and regulatory genes that control the transcription of structural genes. The enzyme activities in the various branch pathways are highly regulated. Transcriptional control play an important role in regulating the overall pathway of flavonoid biosynthesis. This pathway starts from the aromatic amino acid phenylalanine, which is synthesized by the shikimate pathway. Phenylalanine ammonia lyase (PAL) catalyzes the conversion of phenylalanine to cinnamate. The cinnamate 4-hydroxylase (C4H) catalyzes the synthesis of p-hydroxycinnamate from cinnamate and 4-coumarate CoA ligase (4CL) converts p-coumarate to its coenzyme-A ester, activating it for reaction with malonyl CoA which serves as the substrate for anthocyanin production (Jaakola, 2003).

Beld et al. (1989), Charrier et al. (1995) and Bernhardt et al. (1998) conducted detailed studies on the biochemical and molecular aspects of anthocyanin biosynthetic pathway which made the understanding of the pathway easier. The anthocyanin biosynthesis has been comprehensively reviewed in several plant species by a number of investigators (Dooner and Robbins, 1991; Koes et al., 1994; Holton and Cornish, 1995; Mol et al., 1998; Weisshaar and Jenkins, 1998; Buchanan et al., 2000; Winkel-Shirley, 2001).

The precursors for the synthesis of all flavonoids, including anthocyanins, are malonyl-COA and p-coumaroyl-COA. Chalcone synthase (CHS) catalyzes the stepwise condensation of three acetate units from malonyl-COA with p-coumaroyl-COA to yield tetrahydroxychalcone. Chalcone isomerase (CHI) then catalyzes the stereospecific isomerization of the yellow-colored tetrahydroxychalcone to the colorless naringenin. Naringenin is converted to dihydrokaempferol (DHK) by flavanone 3-hydroxylase (F3H). DHK can subsequently be hydroxylated by flavonoid 3'-hydroxylase (F3'H) to produce dihydroquercetin (DHQ) or by flavonoid 3'5'-hydroxylase (F3'5'H) to produce dihydromyricetin (DHM). F35'H can also convert DHQ to DHM.

At least three enzymes are required for converting the colorless dihydroflavonols (DHK, DHQ, and DHM) to anthocyanins. The first of these enzymatic conversions is the reduction of dihydroflavonols to flavan 3,4-cis-diols (leucoanthocyanidins) by dihydroflavonol 4-reductase (DFR). Further oxidation, dehydration, and glycosylation of the different leucoanthocyanidins produce the corresponding brick-red pelargonidin, red cyanidin, and blue delphinidin pigments. Anthocyanidin 3-glucosides may be modified further in many species by glycosylation, methylation, and acylation. There are both species and variety differences in the extent of modification and the types of glycosides and acyl groups attached (Harborne, 1994; Bohm, 1998).

The overview of the flavonoid pathway is presented in Fig. 1

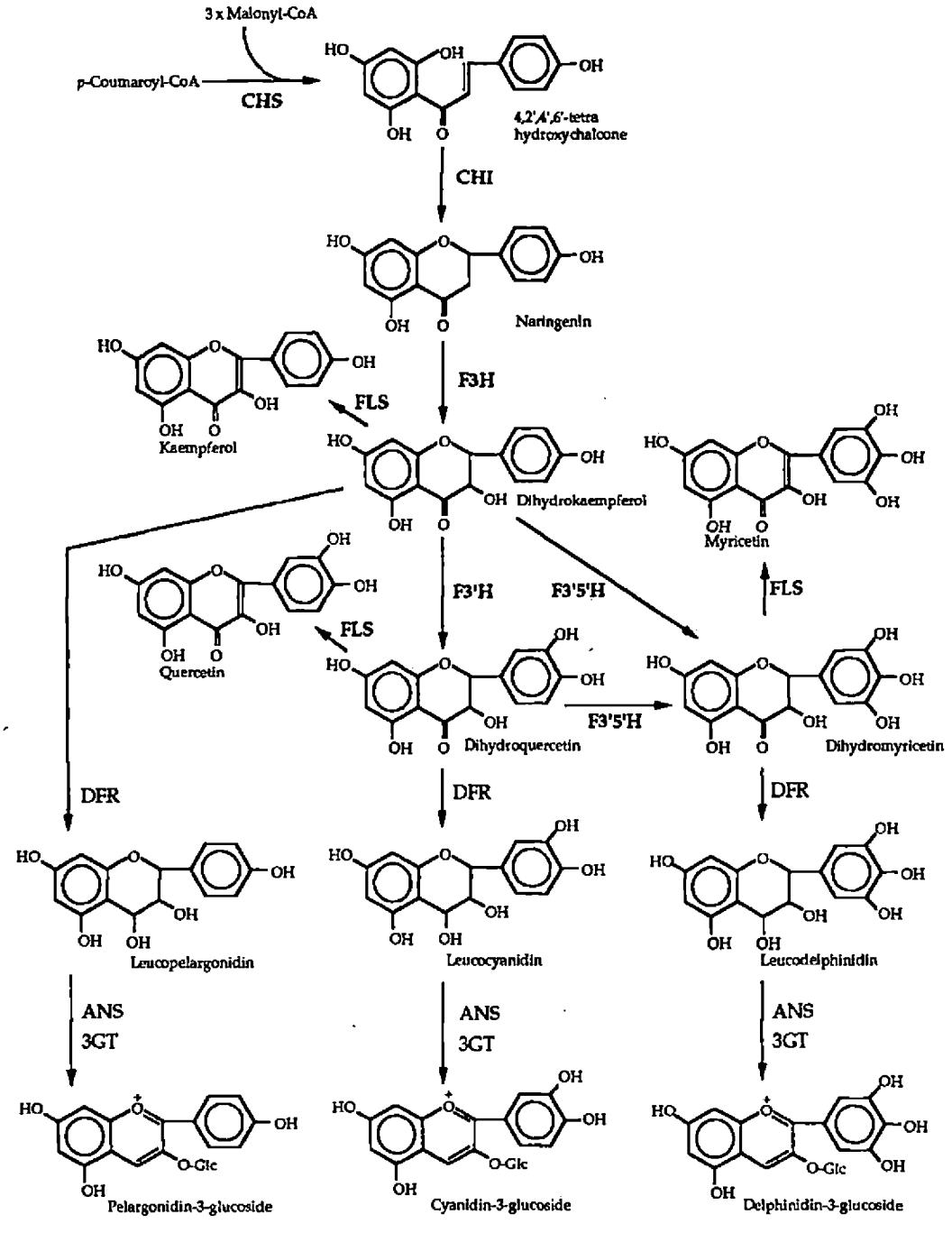

Fig. 1.ANTHOCYANIN AND FLAVONOL BIOSYNTHETIC PATHWAY

2.6 Flavonoid Modification

At every branch 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, glycoslyation, 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 that use frequently as taxonomic markers (Heller and Forkmann, 1993; Harborne, 1994).

Glycosylation, is the most common flavonoid modification process which means transferring a sugar moiety to flavonoid substrates. (Heller and Forkmann, 1993). Several roles have been postulated for glycosylation of various compounds in plants. It allows solubilization of the compounds in water that provides access to the movement of essential nutrients and organic materials within the plant system (Hrazdina, 1988). 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 addition of sugar to the flavonoid aglycone occurs in two ways: it 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-oxy glucosyltransferase to produce a stable, soluble compound (Poulton, 1990).

2.7 Factors Affecting the Expression of Anthocyanin Biosynthesis Genes

The final composition of flavonoids and other phenolic compounds in plants is determined by both genetic and by environmental factors (Robards and Antolovich, 1997). Environmental factors such as temperature, light conditions, reaction of the soil, pH of the vacuolar solution and nature and concentration of sugar may affect the final colour (Brouillard and Dangles, 1994; Brouillard et al., 1997; Mol et al., 1998).

2.7.1 Effect of Light

Anthocyanin biosynthesis was observed to be dependent on the light level the plants were grown under; shade grown plants remained green, while high-irradiance grown plants were dark purple. Light, an important plant developmental signal, influences the accumulation of anthocyanins primarily through the activation of the transcription factors that regulate the flavonoid biosynthetic pathway.

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). Biran and Halevy (1974) showed that covering rose flowers does not inhibit petal growth and pigmentation, whereas covering or removing leaves decreases flower fresh weight and anthocyanin content. Direct illumination of *Eustoma grandiflorum* flowers is not required for *chs* expression and anthocyanin accumulation. These processes are controlled by illumination of the green leaves (Kawabata et al., 1995; Moscovici et al., 1996). Molecular analysis of gene expression in wild-type petunia has shown that light induces expression of the early flavonoid structural genes, as well as those required for flavonol production.

2.7.1.1 Quality of Light

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 hypocotyls (Toguri et al., 1993) and tomato seedlings (Bowler et al., 1994). Blue and red light had similar effects on transcript accumulation, whereas the effect of green light was slightly lower in detached petunia petals (Moscovici et al., 1996).

2.7.1.2 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) studied the effect of light on the expression of anthocyanin biosynthesis genes. He could observe that under weak light conditions, the accumulation of both anthocyanin and mRNAs of all biosynthetic enzymes were lower in leaves of the red form of *Perilla frutescens*.

2.7.2 Temperature

Temperature is one of the main external factor affecting anthocyanin accumulation in plant tissues: low temperatures enhances the accumulation whereas elevated temperature suppresses the accumulation of anthocyanin content. In addition to its effect on anthocyanin synthesis, temperature may also affect pigment stability, with elevated temperatures causing increased degradation. 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.

2.7.3 Effect of pH

A unique feature of anthocyanins is that their molecular structure is pH dependent. At any given pH, an equilibrium containing up to four different molecular structures can occur. The red flavyliumcation is the dominant form at low pH, whereas other forms such as quinonoids, hemiketals and chalcones dominate at higher pH. The equilibrium present in any given situation is likely to have a major effect on anthocyanin bioavailability and bioactivity.

Change of pH during transport of anthocyanidin glucoside into vacuoles is a critical factor determining the color of anthocyanin (Saito and Yamazaki, 2002). Anthocyanins gradually change from red to blue-red, purple-blue and green to yellow as the pH increases.

The petal color of blue morning glory, *Ipomoea tricolor* cv. Heavenly Blue, changes from purplish red in the bud to blue in the fully open flower. The increase of vacuolar pH in the petals during flower opening is due to active transport of Na+ and/or K+ from cytosol into vacuoles through a Na+(K+)/H+ exchanger NXH1 (Yoshida et al., *1997*)

2.7.4 Sugars

Flowers and fruits of most plants are heterotrophic and therefore require imported carbohydrates for their development (Halevy, 1987). Increased sucrose concentration was found to enhance petal growth and pigmentation in detached flowers of rose (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; Bieleski, 1993; Clement et al., 1996).

Weiss and Halevy (1989) studied the mechanism by which sugars regulate petal growth and pigmentation in petunia flowers. When detached petunia corollas were grown *in vitro*, they elongated and became pigmented only in the presence of sucrose and GA3 in the light.

The possibility that sugars play a signaling role in the activation of *chs* expression is supported by reports showing that in *Arabidopsis* and soybean leaves, sugars regulate *chs* expression directly (Tsukaya et al., 1991; Sadka et al., 1994). Since the increase in *chs* expression in the developing petunia corolla coincides with an increase in hexose levels, it was suggested that *chs* is regulated directly by changes in intracellular sugar levels (Tsukaya et al., 1991).

The promotive effect of sugars on petal growth and pigmentation seems to be a general phenomenon. However, it is still not clear whether sugars act in all cases as specific signaling molecules to promote gene expression, or *via* other mechanism (Weiss, 2000).

2.8 Molecular characterization and expression analysis of anthocyanin biosynthesis genes

Anthocyanin biosynthesis has been extensively investigated in several plant species. Two classes of genes are required for anthocyanin biosynthesis; the structural genes encoding the enzymes that directly participate in the formation of anthocyanins and the regulatory genes that control the transcription of the structural genes. The structural and regulatory genes of this metabolic pathway have been cloned and their spatial and temporal expressions were studied (Piero et al., 2005). Major works done on the three key structural enzymes of this complex pathway is described below.

2.8.1 Chalcone Synthase (CHS)

Chalcone synthase (CHS) is a plant-specific polyketide synthase that catalyze the condensation of 4-coumaroyl-CoA with three C2 units from malonyl-CoA to produce naringenin chalcone, a tetraketide which is the central intermediate in the biosynthesis of flavonols, flavones, isoflavonoids and anthocyanins (Buchanan et al., 2000).

Kreuzaler et al. (1983) was successful in isolating the first gene, chalcone synthase from the flavonoid biosynthetic pathway. He determined the nucleotide sequence of an almost complete cDNA copy of chalcone synthase mRNA from cultured parsley cells (*Petroselinum hortens*). The cDNA copy comprised the complete coding sequence for chalcone synthase, a short A-rich stretch of the 5' non-coding region and the complete 3' non-coding region including a poly(A) tail. The parsley *chs* clone was also used as a molecular probe to isolate clones of two different CHS genes from petunia (Reif et al., 1985).

A *chs* gene from snapdragon was isolated by hybridization to the parsley CHS clone (Sommer and Saedler, 1986). The snapdragon genome contains only one CHS gene.

Studies by Koes et al. (1989) revealed that in petunia, chs comprised of a multigene family consisting of 10 *chs* genes in which only one gene was expressed to high levels in petal tissue.

Napoli et al. (1990) attempted to over express chalcone synthase in pigmented petunia petals by introducing a chimeric sense *chs* gene into petunia plants, where both endogenous and the introduced CHS genes were suppressed and resulted in variation in phenotypes.

It has been reported that the introduction of antisense gene in petunia can inhibit flower pigmentation through reduction of *chs* gene expression, which led to the appearance of unusual pigmentation patterns such as even distribution, variegation in sectors and ring pigmentation (van der Krol et al., 1988; 1990).

Sparvoli et al. (1994) isolated genes involved in flavonoid and stilbene biosynthesis from grape (*Vitis vinifera* L.). Clones coding for phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), leucoanthocyanidin dioxygenase (LDOX) and UDP glucose:flavonoid 3-O-glucosyl transferase (UFGT), were isolated by screening a cDNA library, obtained from mRNA from seedlings grown in light for 48 h using snapdragon (*Antirrhinum majus*) and maize heterologous probes.

Jae-Heung Jeon et al. (1996) reported two different full-length nucleotide sequences of cDNAs encoding *chs* multigene family from *Solanum tuberosum* L. (Accession No. U47739 and U47740)

Reddy et al. (1996) identified the presence of a chalcone synthase (CHS) protein in rice seedlings and its developmental stage-specific expression by western analysis. The chalcone synthase of rice was found to be immunologically similar to that of maize. A rice cDNA clone, Os-chs cDNA, encoding chalcone synthase, isolated from a leaf cDNA library of an indica rice variety Purpleputtu has been mapped to the centromeric region of chromosome 11 of rice.

Wha-Shin Hsu et al. (1997) isolated and characterized cDNA encoding chalcone synthase homolog from *Phalaenopsis* (Accession No. U88077). Based on the results of Southern analysis, a family of *chs* genes containing at least 10 complete members exists in the *Phalaenopsis* genome. This type of multigene organization has also been observed in *Ipomoea* (13 *chs* genes, Durbin et al., 1995) and *Gerbera hybrida* (3 *chs* genes, Helariutta et al., 1995).

Rosati et al. (1997) isolated partial gene from petal tissues of *Forsythia intermedia* by RT-PCR. Northern blot analysis was carried out to study the expression pattern of the gene. They transformed colour mutant plants with these genes but the transformants did not accumulate any anthocyanin. They concluded that other genes or regulatory factors should be considered responsible for the lack of anthocyanin production.

Durbin et al. (2001) analyzed the mobile element dynamics in seven alleles of the chalcone synthase D locus (CHS-D) of the common morning glory (*Ipomoea purpurea*) in the context of synonymous nucleotide sequence distances for CHS-D exons by using a nucleotide sequence of CHS-D from the sister species *Ipomoea nil* (Japanese morning glory). They could determine the relative frequency of insertion and loss of elements within the CHS-D locus between these two species.

CHSs have been cloned and sequenced from more than 40 plant species (Abe et al., 2001).

Studies conducted by Jaakola (2003) in bilberry indicated that the expression of CHS was observable throughout fruit development but was reduced with later stages. cDNA fragment of CHS was isolated using RT-PCR. Nucleotide sequence of the fragment exhibited 93 percentage identity to the corresponding genes from other species.

In maize, CHS has been shown to be rate limiting for anthocyanin production (Coe and Neuffer, 1977; Dooner, 1983), although it is not limiting in *Antirrhinum* (Sommer et al., 1988).

Nakamura et al. (2005) demonstrated RNAi suppression of the anthocyanidin synthase gene in *Torenia hybrida* and obtained white flowers with higher frequency and better stability than antisense and sense suppression.

Mudaligae et al. (2005) has isolated and characterized the expression patterns of genes encoding CHS and DFR from a cyanidin-accumulating Dendrobium. Northern analyses indicated that both genes are expressed in all developmental stages of flowers, with highest expression in medium-sized buds. RT-PCR analyses showed that DFR expression was confined to floral tissue while CHS was expressed in floral and vegetative tissues but not in pseudobulbs.

2.8.2 Dihydroflavonol 4- reductase (DFR)

Dihydroflavonol reductase (DFR) is another key enzyme involved in anthocyanin biosynthesis and proanthocyanidin 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 dihydroflavonols to 3, 4-*cis*-leucoanthocyanidins, the immediate precursors of the anthocyanidins (Gollop et al., 2002).

The crucial role of this gene in anthocyanin biosynthesis has been demonstrated by transformation of petunia with a heterologous DFR gene, which led to novel or increased flower pigmentation (Meyer et al., 1987; Helariutta et al., 1993; Tanaka et al., 1995).

DFR genes have been isolated from several higher plants. Beld et al. (1989) isolated a nearly full size cDNA clone (1.5 Kb) from corolla specific cDNA library. Northern blot analysis of mRNA showed that mutants failed to accumulate detectable amounts of DFR mRNA.

The major genes for the enzymes in the anthocyanin biosynthesis pathway have been cloned in grape (Sparvoli et al., 1994), and the spatial and temporal pattern of expression of these genes in grape has been studied (Boss et al., 1996).

The expression during flower development of the gene coding DFR enzyme was investigated in the floral organs of *Forsythia intermedia* cv. Spring Glory by Rosati et al. (1997). Competitive PCR assay was used to quantify mRNA levels in petal tissues. They isolated a putative cDNA clone using RT-PCR.

Moyano et al. (1998) isolated a cDNA clone encoding a putative dihydro flavonol 4- reductase gene from fruit tissues of strawberry (*Fragaria ananasa*) which showed a high homology to DFR from higher plants. Expression analysis indicated that the gene is predominantly expressed in early stages of fruit development.

Zhuang et al. (1999) reported the isolation of full length cDNA sequence encoding a putative dihydro flavonol 4- reductase gene from rice.

2.8.3 UDP- glucose: Flavonoid -Oxy- Glucosyl transferase (UFGT)

The reaction leading from colourless anthocyanidin to its oxy glucoside is the critical step in the formation of coloured metabolites in anthocyanin biosynthesis (Heller and Forkmann, 1988; 1993).

Glucosyl transferase catalyzes the second half of the reaction, *viz.*, the formation of anthocyanidin 3-glucoside from anthocyanidin. However, the detail of this reaction has not been completely clarified in vitro. From inter-tissue complementation assays conducted by Reddy and Coe (1962) in maize demonstrated that leucoanthocyanidin was first converted to anthocyanidin by anthocyanin synthase and then glucosylated by glucosyl transferase.

Fedoroff et al. (1984) reported the first successful isolation of a UFGT gene from maize. Thereafter, many flavonoid glucosyltransferases have been isolated from different sources and biochemically characterized. These enzymes act on different classes of flavanoids at different positions including the 3, 5, and 7-OH groups (McIntosh and Mansell, 1990; Brugliera et al., 1999; De Vetten et al., 1999).

Yamazaki et al. (1999) reported the isolation of a cDNA encoding 5-GT by mRNA differential display from *Perilla frutescens* var. crispa which shared a very little homology with the sequences of several other glucosyl transferases. They also isolated a homologous cDNA clone from cDNA library of *Verbena hybrida,* using this cDNA as a probe.

Sibhatu (2003) isolated and studied the expression pattern of glucosyl transferase gene from grape fruit using RACE-PCR.

The expression of UFGT was investigated by Piero et al. (2005) in both coloured and uncolored oranges. They could observe that UFGT was totally unexpressed in anthocyanin less cultivars analyzed. They isolated partial, putative clones coding for UFGT by RT-PCR. The sequence showed lower identity value when compared with already existing sequences.

Martin et al. (1991) studied the control of anthocyanin biosynthesis in flowers of *Antirrhinum majus* by differential cDNA cloning. Boss et al. (1996) conducted expression analysis in *Vitis vinifera* and concluded that UFGT was regulated independently of other genes and the major control point in the pathway was later than that observed in maize, petunia and snapdragon.

2.9 Improvement of Ornamentals by Genetic Manipulation

Genetic engineering has been widely used for the improvement of ornamentals for the incorporation of desirable traits. Dolgov et al. (1997) reported an efficient regeneration protocol for successful *Chrysanthemum* transformation. Transgenic plants with *bt* toxin, *rolC*, *chs* and AFP genes were produced. De Jong (2000) established procedure for the genetic modification in *Chrysanthemum* by introducing the *CryI C* gene to create plants resistant to insects. Miroshnichenko and Dolgov (2000) transferred the *rol C* gene sequence under 35S promoter on plasmid pPCV002 in *Agrobacterium* strain GV3101 in *Chrysanthemum*. The transformed lines demonstrated

plants with wider petals and changed forms, dramatic changes in phytohormones content especially, in cytokinin auxin ratio were observed.

Mitiouchkina et al. (2000) transferred the full-length cDNA copy of *chs* gene from *A. majus* in *Chrysanthemum* genome of Parliament variety in antisense orientation. The transgenic lines demonstrated suppression of flower colour compared to control plants. Wang et al. (2004) reported transformation of snowdrop lectin gene for aphid resistance in *Chrysanthemum.* Narumi et al. (2005) reported transformation of *Chrysanthemum* with mutated ethylene receptor genes mDG-ERS1, for reduced ethylene sensitivity.

Deroles et al. (2000) modified the chalcone biosynthesis in *Petunia hybrida* by introducing a *Chs* cDNA from *Medicago sativa* under the control of the 35S CaMV promoter into the white flowered Mitchell line of *Petunia.* The flower color was changed from white to pale yellow. Esposito et al. (2000) reported the introduction of gene *ech-42*, encoding an endochitinase from the antagonist fungus *Trichoderma harzianum*, alone or in combination with the osmotin gene from *Nicotiana tabacum* into a *Petunia hybrida* pure line by the *Agrobacterium tumefaciens* leaf disc system. The transgenic lines revealed a statistically significant reduction of the disease symptoms in comparison with the controls.

Miroshnichenko and Dolgov (2000) reported *Agrobacterium* mediated transformation procedure for carnation using the hygromycin resistance gene (*Hpt*) as a selective agent. Ovadis et al. (2000) used a highly efficient and reliable transformation procedure for carnations. Carnations with colour modifications and altered plant morphology and performance were obtained by introducing an *fht* antisense gene and a *rol C* gene, respectively.

Babu and Chawla (2000) reported *Agrobacterium* mediated genetic transformation in gladioli using gus gene. Loffler et al. (2000) reported the genetic

transformation of gladioli for fusarium resistance using particle bombardment with gold particles coated with a construct harboring the *gus* reporter gene and the *pat* selection gene. Suzuki et al. (2001) reported *Agrobacterium* mediated transformation in lillaceous ornamental plants

Condliffe et al. (2001) reported the *Agrobacterium* mediated genetic transformation in rose cultivars.

Ketsa et al. (2004) studied the role of ethylene in ovary growth of *Dendrobium* 'Pompadour'. Ethylene synthesis was inhibited using aminooxyacetic acid (AOA). AOA delayed the time to wilting of 50 per cent of the flowers. Chan et al. (2005) reported gene stacking on *Phalaenopsis* orchid by double transformation to enhance the resistance of orchids to both viral and bacterial phytopathogens. This is the first report describing a transgenic *Phalaenopsis* orchid with dual resistance to phytopathogens.

2.10 Transformation Using Acc Synthase Antisence Gene

Altvorst et al. (1994) reported that the senescence of flower petals is a highly regulated developmental process which requires active gene expression and protein synthesis. The gaseous phytohormone ethylene plays a critical role in the regulation and coordination of senescence processes. The ethylene biosynthesis path way could be regulated through antisense RNA technology and ethylene response could be blocked. This could improve post harvest longevity of cut flowers like carnation.

Iwazaki et al. (2004) reported *Agrobacterium* mediated gene transformation of Carnation (*Dianthus caryophyllus* L. cv. Nora) plants using ACC synthase antisense (*DC-ACS1*) gene. The transgenes showed suppressed ethylene production during natural senescence as compared with flowers of the non-transformed control. Shaw et al. (2004) reported *Agrobacterium* mediated genetic transformation in *Petunia hybrida* using *boers*, an ethylene receptor sensor gene of *Brassica oleracea.* Transformed plants produced apparently larger flowers.

Theologis et al. (1993) produced transgenic tomato plants expressing antisense RNA of the key enzyme in the ethylene biosynthetic pathway, 1-amino cyclopropane-1-carboxylate (ACC) synthase using the constitutive CaMV 35S and fruit specific E8 promoters. Fruits expressing antisense LE-ACS2 RNA produce less ethylene and failed to ripen. Ju et al. (1994) inserted the *PG* gene for studying the inhibition effects of its antisense RNA on the expression of *PG* gene in transgenic tomato plants. The results suggested that the expression of the anti-*PG* gene effectively inhibited the expression of endogenous PG gene.

Nakatsuka et al. (1997) transformed tomato with ACC synthase antisense gene and observed strong positive feed back in ACC synthase and ACC oxidase gene transcriptional level in tomato fruit, even at the stage with a burst of ethylene production. Liu et al. (1998) reported *A. tumefaciens* mediated transformation of tomato using ACC synthase antisense gene. The amount of ethylene released from transgenic tomato fruits was reduced significantly (30%) of that released by non-transformed controls. The shelf life of transgenic tomato fruits was at least 60 days at room temperature without significant change in hardness and color. Xiong et al. (2003) reported the construction of binary plant expression vector pOSACC in which the double- antisense ACC oxidase and ACC synthase fusion gene was introduced, controlled by fruit-specific 2A11 promoter. The transgenic tomatoes showed the characteristics of prolonged shelf life over 50 days. Ethylene released from the transgenic fruits was reduced significantly to about 9.5 per cent of that released by non-transformed controls.

Laurena et al. (2000) prevented the post-harvest loss in papaya by down regulating ethylene biosynthesis through antisense expression of ACC synthase gene. ACS_2, a good candidate gene of ACC synthase antisense strategies, was used to regulate fruit ripening. The gene was inserted in an antisense orientation in two types of vector constructs for papaya transformation.

2.11 *Agrobacterium* Mediated Genetic Transformation in Orchid

There is substantial interest in the genetic improvement of orchid. Orchids form the largest family of flowering plants with 25, 000 species that are commercially grown (Arditti, 1992). Kuehnle and Sugii (1992) first reported the genetic transformation in orchids. Chia et al. (1994) reported the genetic transformation in orchid using firefly luciferase gene. Knapp et al. (2000) reported the transformation of *Dendrobium* using *bar* gene. Yu et al. (2001) reported the generation of transgenic orchid (*Dendrobium* Madame Thong-In) plants by inoculating thin section explants from PLBs with *Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector that carried the orchid *DOH1* antisense gene.

Liau and Lu (2003) reported for the first time the successful transformation into PLBs of *Oncidium* orchid using *Agrobacterium tumefaciens* strain EHA105 harbouring the sweet pepper ferredoxin-like protein (pflp) which conferred resistance against soft rot disease. Liau et al. (2003) established a transformation procedure via *Agrobacterium tumefaciens* for an important *Oncidium* orchid cultivar. An expression vector containing *hptII* and *gusA* genes driven by the cauliflower mosaic virus (CaMV) 35S promoter was successfully introduced into the PLBs. You et al. (2003) successfully transformed protocorm-like bodies of *Oncidium* orchid using *Agrobacterium tumefaciens*. The pflp gene was used as selection marker and *Erwinia carotovora* as the selection agent, thereby obtaining transgenic plants without the use of an antibiotic selection agent.

Belarmino et al. (2000) reported *Agrobacterium* mediated genetic transformation of *Phalaenopsis* orchid for hygromycin resistance. Men et al. (2004) reported the transformation in *D. nobile* by biolistic bombardment. The plasmid pCAMBIA1301 encoding beta-glucuronidase gene (*gus-int*) and a hygromycin phosphotransferase (*hpt*) gene were introduced into the PLBs. Mishiba and Chin (2005) reported *Agrobacterium* mediated transformation of *Phalaenopsis* by targeting protocorms at an early stage after

germination. Chan et al. (2005) reported the gene stacking on *Phalaenopsis* orchid for resistance against both viral and bacterial phytopathogens. Gene stacking was applied by double transformation. This enabled the expression of dual (viral and bacterial) disease resistant traits.

2.12 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Different techniques such as northern blot, dot blot, RNA protection assay, *in situ* hybridization and RT-PCR can be used to study the expression of specific genes at the mRNA level. Among these RT-PCR offers a high level of specificity and sensitivity and thus it is frequently the method of choice for studies of gene expression (O' Driscoll et al., 1993).

2.12.1 Primer Designing

Primer design is the single largest variable in PCR applications and is the most important factor in determining the result of PCR reactions. Gene specific primers are designed based on the conserved sequences present among the gene of interest (Sibhatu, 2003). Several organisms share conserved regions for anthocyanin biosynthesis genes (Dooner et al., 1991). Multiple sequence alignment programme 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).

Innis et al. (1990) suggested a set of rules for primer sequence design which include, Length of primer (17 – 28 bases), G + C content (50 – 60%), the presence of G or C or CG or GC at (3') end,and a preffered melting temperature between 55-80 °C. It should not have three or more Cs or Gs at the 3' ends, 3' end complementarity and self complementarity. Primer3 software (http://fokker.wi.mit.edu/primer3/.) designs primers for PCR reactions, according to the conditions specified by the user. Primer3 consider

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. All of these conditions are user-specifiable, and can vary from reaction to reaction. The software was originally developed by Rozen and Skaletsky (2000).

Primer design has two essential phases: physical design and selectivity design. Physical design of primers involves the consideration of the factors such as GC content, primer length, annealing and melting temperatures, starting nucleotides and higher order oligo nucleotide structure. These factors are essential to ensure that a primer is able to bind to a template and initiate extension by the polymerase in an efficient, consistent manner. Primer selectivity refers to the ability of a primer to bind to a single location within the initial pool of DNA (Boutros and Okey, 2004).

2.12.2 RNA Isolation

Isolation of good quality total RNA and mRNA free of protein, genomic DNA and secondary metabolite contamination is crucial for cDNA library construction and molecular analysis like northern hybridization and RT-PCR (Liu et al, 1998).

Good preparations of eukaryotic mRNA is necessary to minimize the activity of RNases liberated during cell lysis by using inhibitors of RNases or methods that disrupt cells and inactivate RNases simultaneously (Sambrook and Russel, 2001).

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 rinsed with chloroform (plastic ware). Fedorcsak and Ehrenberg (1966) identified diethyl pyrocarbonate (DEPC) at the concentration of 0.1% in water as a strong inhibitor of RNases. After treatment, the DEPC filled labware were 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).

Blackburn et al. (1977) observed that many RNases bind tightly to a protein isolated from human placenta to form an enzymatically inactive complex. This protein can be isolated and used as RNase inhibitor. Martynoff et al. (1980) reported that RNase inhibitor from human placenta required sulfadryl reagents such as Dithiothreitol (DTT) for maximal activity. The RNase inhibitor protein would not interfere with reverse transcription.

Proteins dissolve readily in solutions of potent denaturing agents such as guanidine HCL and guanidine thiocyanate (Cox, 1968). RNases are inactivated by 4M guanidine thiocyanate and reducing agents such as β-mercaptoethanol (Sela et al., 1957). This combination can be used to isolate intact RNA from tissues rich in RNases (Chirgwin et al., 1979).

Numerous standard protocols have been developed for the effective isolation of high quality RNA suitable for functional genomics based experiments (Chomezynski and Sacchi, 1987; Logemann et al., 1987; Ainsworth, 1994; Sambrook and Russel, 2001). Efficiency of these methods varies with the type of tissues. Most published protocols, for RNA isolation, have used strong protein denaturants and guanidine / guanidium salts for RNAse inactivation. Liu et al. (1998) found that the guanidine based method is useful for the isolation of RNA from banana leaf and root tissues but it fails to recover RNA from banana fruit tissues.

Studying gene expression in fruit tissues is technically complicated due to the presence of large quantities of polysaccharides and polyphenolic compounds that accumulate in this tissue. These compounds often co-precipitate and contaminate the

RNA during extraction affecting both quantity and quality of RNA isolated (Asif et al., 2000, Logemann et al., 1987).

Ainsworth (1994) suggested that 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.

Liu et al. (1998) have isolated good quality RNA at high temperature (80° C) from fruit tissues of banana at several ripening stages. Method developed by Schneitz (2000) utilizes hot phenol maintained at 80 °C for isolation of total RNA from plant tissues rich in polysaccharides.

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 LiCl precipitation.

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 (Chomezynski and Sacchi, 1987), resulted in total RNA that was contaminated with large quantities of polysaccharides and polyphenolic compounds. The third protocol which was a modification of several other RNA isolation protocols from plants (Chang et al., 1993; Salzman et al., 1999; 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.

2.12.3 mRNA Purification

In contrast to rRNA and tRNA, mRNA carry 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; 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 Persson, (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 % ethanol. Poly(A)$^{+}$ RNA was then eluted by heating the filters at 70°C for five minutes in water.

Elution with streptavidin coated paramagnetic polystyrene 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 biotinylated 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.

2.12.4 RT-PCR

RT-PCR is an increasingly popular method for quantitative analysis of gene expression because it 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 cDNA. 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 Moloney strain of murine leukemia virus are commonly used for reverse transcription reaction. Kotewicz et al. (1988) reported that the enzyme coded by AMV has a powerful RNaseH and endonuclease activity that can cleave the RNA-DNA hybribs and restrict the length of cDNA. Gerard et al. (1997) found that the murine enzyme was better suited for RT-PCR because it lacked RNaseH 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.

3. Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Isolation and characterization of cDNA encoding chalcone synthase gene from flower buds of orchid *Dendrobium* variety Sonia 17" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram, during the year 2005 to 2007. Details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3.1 Materials

3.1.1 Plant Material

Immature flower buds of orchid *Dendrobium* variety Sonia 17 were used for the isolation of messenger RNA since the expression of chalcone synthase will be maximum at the very early stages of anthesis according to (Rosati et al., 1997). The plants were purchased from an authenticated orchid grower and were maintained at the farm of college of Agriculture, Vellayani, Thiruvananthapuram. Flower buds were collected prior to the conduct of the experiment. (Plate 1 and 2).

3.1.2 Gene specific Primer

A primer is a short synthetic oligonucleotide that is used in many molecular techniques from PCR to DNA sequencing. The primer is designed to have a sequence, which is the reverse complement of a region of template, or target DNA to which it anneals.

3.1.3 Other Materials

mRNA purification kit (KT 80, Bangalore GENEI), RT-PCR kit (KT 74, Bangalore GENEI), chemicals and other lab equipments such as programmable Thermal Controller (PTC 100, MJ Research), Centrifuge, Spectro Photo Meter, Gel documentation system etc.

3.2 Primer Designing

Primers for chalcone synthase gene were designed using Primer3 software (Rozen and Skaletsky, 2000). Forward and reverse primers were designed based on conserved sequence of the chalcone synthase gene identified in other crops. 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(Thomson et al., 1997) multiple alignment program. The conserved regions were then selected and were used for designing primers with Primer3.

Certain parameters were fixed for primer designing. The primer length varied from 21 nt – 36 nt with GC content greater than 50.0 percent. The 3' region of the primer ends with guanine or cytocine except for the reported primer. The difference in melting temperature between forward and reverse primer was a maximum of five in all the combinations.

Primers (Af and Ar) were designed based on the conserved region of the gene chalcone synthase in *Oryza sativa* with accession number X89659. Primers (Bf and Br) were designed using the sequences of *Fragaria ananassa* chalcone synthase gene (Accession number AY997297). The other two primers (Cf and Cr) and (Df and Dr) using the conserved region of the gene in orchid. One reported primer set was also included in the experiment. Primers (Ef and Er) were selected from the published work of *chs* gne in Phalaenopsis orchid (Ying ying han et al., 2005)

3.2.1 Primer analysis

The primers were analyzed by BLAST N (Altschul et al.,1997) to find the binding region of the primers in different organisms. Oligonucleotide properties calculation programme (www.basic.northwestern.edu/boitools/oligocalc.html) was used to find the 3'complementarity, hairpin formation and self annealing of the primer pairs.

3.2.2 Synthesis of primer

The primers were synthesized and supplied by Integrated DNA technologies (IDT), USA.

3.2.2.1 Isolation of Total RNA

Isolation of good quality total RNA and mRNA free of protein, genomic DNA and secondary metabolite contamination is the first and often the most critical step in gene isolation.

The major problem in any RNA related work is contamination by ribonucleases. Ribonucleases (RNases) are very stable enzymes that catalyze the degradation of RNA molecules and generally require no cofactors to function. They cut single stranded RNA at designated base sites. They can temporarily be denatured under extreme conditions but it readily renatures. They can withstand temperatures up to 100°C, extreme pH and remain active for weeks. They can survive autoclaving and other standard methods of protein inactivation. Therefore all materials used in extraction were kept free of RNAse contamination.

To reduce RNase contamination, following precautions were taken during the entire course of work.

1. Centrifuge tubes, micro tips, mortar, pestle, spatula and glassware were treated with 0.1 per cent diethyl pyro carbonate (DEPC), a strong inhibitor of RNase. DEPC reacts with a histidine residue at the active site of RNases, rendering them inactive.

Preparation of DEPC solution

0.1% solution of DEPC was prepared by adding 1 ml of DEPC to one litre of distilled water and mixed well. The labwares were then soaked in DEPC water in separate autoclavable containers and covered with aluminium foil and incubated at dark for 8 - 12 hours in an isolated area. After incubation, the DEPC 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. Incomplete removal of DEPC may result in carboxymethylation of the purine residues of RNA or single stranded DNA, resulting in a loss of biological activity. After autoclaving the equipments were baked for ten hours at 60°C.

2. Sterile gloves were worn during the entire course of work to reduce RNase contamination.
3. The working area was cleaned with 70% ethanol before the conduct of experiment.
4. Non-disposable plasticware were also treated to inhibit RNases. These were first rinsed in chloroform, followed by DEPC treated, autoclaved water, to inhibit RNase contamination.
5. Water and other reagent solutions were treated with 0.1 percent DEPC and autoclaved prior to use. DEPC treated autoclaved water was used to prepare solutions.
6. Electrophoresis gel boxes were treated with three percent hydrogen peroxide (v/v) for 10 -15 minutes and then rinsed with RNase free water before the run.

PROTOCOLS

Four standard protocols were tried for the isolation of total RNA from immature flower buds of orchid. The first method tried was using mRNA purification kit of GENEI (Bangalore). But the quality of RNA obtained was not satisfactory. The second method used was that developed at Rubber Research Institute of India which utilizes 3M lithium chloride for precipitation of RNA from lateciferous tissues. Extraction of total RNA with phenol chloroform followed by ethanol precipitation was also checked. Fourth and successful method tried was that developed by Schneitz (2000) which utilizes hot phenol for the isolation of total RNA. This gave good quality total RNA from orchid floral tissue. Freshly harvested immature flower buds of orchid were used to get good quality RNA. The procedure followed for the isolation of total RNA is as follows.

1. One gram plant tissue was ground into fine powder in liquid nitrogen along with a pinch of PVPP in a pre-treated mortar and pestle.
2. Finely ground powder was then transferred quickly to a 2ml micro centrifuge tube containing 2ml extraction buffer /phenol mixture (1:1) maintained at 80°C, followed by addition of 50µl of β Mercaptoethanol (Appendix I).
3. The tubes were kept at 80°C for 3 minutes by placing them in waterbath maintained at the above temperature.
4. The contents were then vortexed to avoid isolation of genomic DNA.
5. The mixture was centrifuged at 10000 rpm for 15 minutes in a refrigerated centrifuge at 4°C.
6. Supernatant was then transferred to a new tube to which an equal volume of chloroform was added.
7. The tubes were shaken at room temperature for 30 minutes in an incubator shaker.
8. The mixture was then centrifuged at 12,000 rpm for 20 minutes.
9. The upper phase was transferred to a new tube and 1/3 volume 8 M LiCl was added to it, to bring the final LiCl concentration to 2 M.
10. The tubes were incubated overnight at -20°C to precipitate RNA.
11. The precipitated RNA was pelleted at 10,000 rpm for 15 minutes in a refrigerated centrifuge.
12. Pellet was dissolved in 0.5ml 2 M LiCl and was centrifuged for five minutes at 10,000 rpm.
13. Recovered RNA was then washed twice with 70 per cent ethanol.
14. To purify further, the pellet was again washed with absolute ethanol and air dried for 15 to 30 minutes.
15. The dried pellet was dissolved in 50 µl RNase free water and stored at -80°C.

3.2.2.2 Quantification of RNA

The assessment of RNA integrity is a critical first step in obtaining meaningful gene expression data. UV Spectroscopy method was used for assessing RNA concentration and purity. The absorbance of diluted RNA samples was measured at 260 and 280 nm (SPECTRONIC GENESYS 5). The RNA concentration was calculated using

Beer –Lamberts equation. Sample readings are made in quartz cuvetts. Blank reading was also done at 320 nm followed by readings at 260 and 280nm.

Since an absorbance value of one corresponds to approximately 40 µg/ml for RNA, the RNA concentration in the sample was calculated as follows

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

A_{260} = Absorbance at 260 nm

RNA purity was determined by the ratio taken between A_{260}/A_{280} readings.

3.2.2.3 Analysis of cDNA using Agarose Gel Electrophoresis

The most common method to assess the integrity of total RNA is to run an aliquot of the RNA sample on a denaturing agarose gel stained with ethidium bromide. The integrity and size distribution of the total RNA was determined by running 5µl aliquots of RNA on 1.4 per cent agarose gel. Electrophoresis was carried out at 40 V for six hours in 1X TAE buffer (Appendix II). The gel was stained with ethidium bromide and analyzed using gel documentation system (BIO-RAD, USA).

3.2.2.4 Purification of mRNA from Total RNA

Purification of mRNA from total RNA isolated was carried out using mRNA purification kit (KT 80, Bangalore GENEI). This involves three steps.

1. Binding

   The total RNA was added to the vial of oligo $d(T)_{25}$ silica and mixed well by gentle tapping. The volume was then made up to 200 µl with DEPC water. The tube was incubated at 65°C for five minutes with intermittent mixing. After incubation 20 µl of 5M NaCl was added to the tubes and again incubated for ten minutes at 37°C. Ten minutes later, the contents were centrifuged at 10,000 rpm for ten minutes at room temperature. The supernatant was then carefully removed with a micropipette without disturbing the pellet.

2. Washing

The pellet was suspended in 200 µl of wash buffer by gentle mixing. The suspension was centrifuged at 10,000 rpm for ten minutes at room temperature to pellet the RNA. Supernatant was discarded by removing with the help of a micropipette without disturbing the pellet. This step was repeated twice.

3. Elution

The pellet was again suspended in 25 - 50 µl of TE buffer and incubated at 65°C for five minutes. The suspension was then transferred onto the spin column using micropipette. The column was placed into the sterile 1.5 ml eppendorf vial. The vials were centrifuged at 5000 rpm for five minutes at room temperature to recover mRNA.

3.2.2.5 RT - PCR

RT-PCR (reverse transcription-polymerase chain reaction) is a sensitive technique for mRNA detection and quantitation. 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. To avoid any RNase contamination in the buffers and containers used in RNA preparation, pretreatment with DEPC followed by autoclaving/ baking was done. All materials used in the experiment were autoclaved prior to use.

3.2.2.5.1 Materials

The materials required for RT-PCR reaction include, good quality mRNA, oligo $d(T)_{18}$ primer, RNase inhibitor, 5X RT buffer, 30m M dNTP mix, M-MuLV reverse transcriptase, 0.1M DTT, 10X PCR buffer, *Taq* DNA polymerase, Magnesium chloride and gene specific primers.

3.2.2.5.2 First Strand cDNA Synthesis

In the first step of RT-PCR, called the "first strand reaction," complementary DNA is made from a messenger RNA template using dNTPs and an RNA-dependent DNA polymerase, reverse transcriptase, through the process of reverse transcription. The cDNA was prepared from 5ul of mRNA, which was reverse transcribed by M-MLV reverse transcriptase (Bangalore GENEI) from an oligo $d(T)_{18}$ primer using standard methods in a reaction volume of 20 µl.

Procedure

9.5 µl of mRNA sample (10 -100ng) was added to a sterile PCR tube. After mixing gently, 1µl oligo $d(T)_{18}$ primer was added to the mixture. 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.

The reaction mixture was prepared by adding the following

Component	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

The solution was mixed well and incubated first at 37°C for one hour and then at 95°C for two minutes to denature RNA – cDNA hybrids. After incubation, a brief spin was given and the tubes were quickly placed on ice.

3.2.2.5.3 PCR Amplification

Gene specific primers of chalcone synthase were used to carry out amplification of cDNA product. A 25µl reaction mixture was set in the Programmable Thermal Controller PTC-100 (MJ Research, USA). The reaction was set as following.

Item	Volume (µl)
cDNA product	6.0
10x PCR buffer	2.5
30mM dNTP mix	2.0
Forward primer (100ng/µl)	0.5
Reverse primer (100ng/µl)	0.5
Taq DNA polymerase (3U /µl)	1.5
Magnesium chloride	0.5
Sterile water	11.5
Total	25.0

The PCR was carried out by denaturing the PCR mix at 95°C for two minutes followed by 30 cycles of denaturation at 94°C for 60 seconds, annealing for 52 seconds and extension at 72°C for two minutes. Final extension at 72 °C for seven minutes was given. Annealing temperature was changed based on the Tm of primers as shown below.

Primers	**Annealing Temperature (^{0}C)**
Af	55.50
Ar	56.00
Bf	50.70
Br	55.20
Cf	57.60
Cr	55.70
Df	56.30
Dr	55.70
Ef	65.20
Er	61.60

The PCR conditions for the reported primer set was 94°C for five minutes, 94°C for 60 seconds, 55°C for 60 seconds and 72°C for two minutes with 35 cycles. Final extension at 72 °C for ten minutes was given.

Control reactions were carried out cDNA to distinguish the target product from non target products and primer dimer formation. After PCR, gel electrophoresis was carried out on 1.4 per cent low melting agarose gel in 1X TAE buffer at 35 V for five hours.

3.2.2.5.4 Elution

Low melting gel was prepared by casting the gel at 4°C. In the case of non specific amplification, bands of the approximate expected sizes were excised from the gel and gel elution was carried out.

Low melting agarose gel slice containing excised cDNA was placed in a 1.5 ml micro centrifuge tube. The tubes were kept at 65°C in order to melt the agarose gel slice. One volume of TE saturated phenol was added to the gel and mixed well .The tubes were vortexed for 30 seconds. The samples were centrifuged in a micro centrifuge tube at 12,000 rpm for five minutes at room temperature to separate the phases. The aqueous phase was then transferred to a fresh tube, followed by precipitation with 2.5 volumes of absolute ethanol. The pellet was finally air dried and dissolved in sterile water.

3.2.3 Sequencing

Sequencing was done at Banglore GENEI. Sequence analysis was done in automated ABI 31000 Genetic analyzer that uses fluorescently labeled primers. Sanger's sequencing method was used.

3.2.5 Data Analysis

Similarity searches were carried out in the NCBI database using nucleotide – nucleotide (BLASTN) and translated query *vs.* protien database (BLASTX) searches to identify homologous sequences from other species.

Plate 1: *Dendrobium* variety Sonia 17

Plate 2: Immature flower buds of *Dendrobium* variety Sonia 17

4. Results

4. RESULTS

The result of the study entitled "Isolation and characterization of cDNA encoding chalcone synthase from orchid *Dendrobium* variety Sonia 17" was carried in the Department of Plant Biotechnology, College of Agriculture, Vellayani are presented below. Reverse transcription - polymerase chain reaction was carried out to study the expression of gene.

4.1 MOLECULAR ANALYSIS

4.1.1 Primer Designing

Heterologous gene specific primers for the gene chalcone synthase were designed (Table 1) using Primer3 software. Clustal X 1.81 multiple alignment programme was used to identify the homologous regions of the gene. Based on the conserved sequence of the gene, chalcone synthase in other crops, forward and reverse primers were designed and more than one primer combinations were obtained for the gene.

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.

Primers designed using chalcone synthase sequences obtained from rice strawberry and orchid were selected for amplifying chalcone synthase gene. One reported primer from *Phalaenopsis* orchid was also included in the study. The primers were first tested for PCR amplification using genomic DNA of orchid. PCR conditions were standardized for optimum results.

Table 1. Primers designed

Sl. No	Gene	Name of Primer	Tm	GC%	Primer length	Sequence of primer	Source plant
1	CHS	Af	55.50	52.38	21	5'GACAAGTCGCAGATCAGGAAG3'	*Oryza sativa*
		Ar	56.00	52.38	21	5'CTACCAACACACGGGAAAAGG3'	
2	CHS	Bf	50.70	52.38	21	5'CACCACCCAACTGTATTGACC3'	*Fragaria ananasa*
		Br	55.20	52.38	21	5'CCAAATAGAACACCCCACTCC3'	
3	CHS	Cf	57.6	57.1	21	5'GTGCCGACTACCAACTCACTC3'	*Orchid*
		Cr	55.7	52.3	21	5'GCCTTTATAGCTCCACCCAAC3'	
4	CHS	Df	56.3	52.3	21	5'TGGABCTGACCCGGACTTGAC3'	*Orchid*
		Dr	55.7	52.3	21	5'GCCTTTATAGCTCCACCCAAC3'	
5	CHS	Ef	65.2	47.2	36	5'GCTCTAGAATGCAAGGAGAAATTCCAGCGCATGTGT3'	*Orchid*
		Er	61.6	56.0	25	5'GGGAGCTCAACAGAACACCCCATTC3'	

4.1.2 RNA Isolation

Immature flower buds of orchid Dendrobium variety Sonia 17 were used for the isolation of total RNA. Four different protocols were tried. Since orchid floral tissue contains large quantity of polyphenols and mucilaginous, extraction of good quality total RNA was difficult. The protocol which utilized hot phenol was found to be most effective for the extraction of RNA from floral tissues of orchid.

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 28S rRNA and 18S rRNA with no genomic DNA contamination. The intensity of 28S rRNA band was approximately double the intensity of 18S rRNA band (Plate 3). The yield of RNA was 80 - 200 $\mu g\ g^{-1}$ of the tissue.

The quality of RNA was good with an A_{260}/A_{280} ratio ranging between 1.6 - 2.0.

4.1.3 RT -PCR

Reverse transcription polymerase chain reaction was carried out using the selected primers for gene specific amplification. The primer designed from mRNA for chalcone synthase from rice gave better amplification of cDNA isolated compared to that obtained using primers based on strawberry. The cDNA amplified with primers Af & Ar and Bf & Br showed single band of approximately 400 bp and 700 bp respectively. Approximately 200 bp was obtained when cDNA amplified with primers Df and Dr (plate4).

Primer dimers were visualized in the cDNA samples during amplification. For the optimization of the reaction, the concentrations of the primers were lowered and adjusted the annealing temperature by increasing it.

4.1.4 Sequencing

Complementary DNA amplified with chalcone synthase specific primer after sequencing was found to have a size of 460, 509 and 210 bp in orchid. (Table 2).

4.1.5 Similarity search

Homology search was done with BLASTN (nucleotide with nucleotide search) and BLASTX (nucleotide to protein) program of NCBI to characterise the cDNA.

The results of the nucleotide to nucleotide search (BLASTN) of the cDNA clone of orchid, amplified using chalcone synthase specific primer Af and Ar, showed maximum similarity with cDNA clone 5', mRNA sequence of flower bud of *Phalaenopsis violacea* (GI: 110663973, CK858452.1) and cDNA clone 5', mRNA sequence of *Phalaenopsis equestris* (GI: 109152803, CB034051.1)Lambda ZapII cDNA Library with an Expectation value (E value) of 5.6 and a maximum score of 28.3. (Fig 2).

The translated query vs. protein database (BLASTX) search revealed similarity with a hypothetical protein, maturase K (*Aerangis kirkii*) (GI: 71913027, GB AAZ53016.1) with an E value of 2.2 and a bit score of 24.6. (Fig 3).

The results of the nucleotide to nucleotide search (BLASTN) of the cDNA clone of orchid, amplified using chalcone synthase specific primer Bf and Br, showed maximum similarity with cDNA clone 5', mRNA sequence of flower bud of *Phalaenopsis violacea* (GI: 110663423, CK857808.1) with an E value of 1.8 and a maximum score of 30.1 and flower bud of *Phalaenopsis equestris* (GI: 109154466, CK855950.1) with an E value of 6.2 and a maximum score of 28.3. (Fig 4).

The translated query vs. protein database (BLASTX) search revealed similarity with a hypothetical protein, LFY-like protein OrcLFY (*Serapias lingua*)

(GI: 27544595, DBJ BAC55082.1) with an E value of 2.0 and a bits score of 25.0. (Fig 5).

The results of the nucleotide to nucleotide search (BLASTN) of the cDNA clone of orchid, amplified using chalcone synthase specific primer Df and Dr, showed significant similarity with cDNA 5', mRNA sequence of *Ipomoea batatas* (GI: 114781594, EE878220.1) with an E value of 5.9 and a maximum score of 37.4. (Fig 6).

The translated query vs. protein database (BLASTX) search revealed similarity with a hypothetical protein, retrotransposon protein, putative, unclassified (*Oryza sativa* (japonica cultivar-group)) (GI: 110288992, GB ABB47352.2) with an E value of 7.1 and a bits score of 28.9. (Fig 7).

Table 2. Sequence obtained after amplifying with chalcone synthase specific primer

Sl. No.	Sequence Length (bp)	Sequence (5' – 3')
1	460	CTAAGATGGGTCAGCACAGCTCGCGTTACGAATGGGGCCAGCA ATTGTGGATTTTTCGTGCTAGGATTACATTTACTTGTACTTGGAG AGCCAAATTGGCTTGTCCAATTATTCTTGTAATTATGGGATTTG GACAGCGGTACTGGCTACCTCTTCTCTTCTGCATTTGCTTGGATT CCCTGTATAGGTTTTTTATAACACATTTGCCCTCACCATTGTTTT TCCCCTGGCTTATATTTTCAGCCCGAATAAGGAATGGGACCAGC ATTTCTTTGCCACTTGGAGTTTTGAATATTTTATTTACCCCTTTG AACTATTCCCAGCTATTATGGAGTAAGACGTTAAGAATCCAACG ACCCGCCCAACTCCTGAAGGGTGCCCCAATCCTAGCGTGTGAGG GCCTTTAAACATCTCTTAGTTCCACTTGCCTTCTCAAATTTTTTA AGACTGGGGCCAACAG
2	509	CAAGTTCATCCGTTCTCCATTTGTACATTTCTTTCTAGCCTTTCT AGTTAAATTTTTCCCCCCTATGATCGGAGGAAATCTCCACTGTT CAAACCCAATCCAATGCCTCAATTGAATAACCTCTCTAAAATCA AAATGCAAATGGTTCTCAAATCTTCCAATGATACTTATGAGATA TAGAATTACATTCCTTCAGAGAATTCCTTCTTTCTACCTCTAATT TACCTTTGACATAATTTTTCTTCAAATCTATTCGAGATTTAGATT AACTTTATTTTTCCGTATAAATGAGTGGTGAAGGAAATTTTCAT GTTGTTGACTATTTGAATTTTAAATTCCCTTCTATTTTTTATGGA TATAAGGATAAGTTAGCAAAAATCAAAGAAATCAAAATTTTAA AATCAAAAACTCAATCACATAAGCACACTTTTGTGAATCCCCAT ATGGTTCTTACTGATTGACTCTTTAATCTTAAATTTTAATGGCTT ATTTTACCCCCCTGCCATATG
3	210	CATAATAGCTTATCTAGCCAGTAACGAAGCTGCATCCTAGTCTA GGCGCAGGATCTGACGGGTCCTCGAGTGGCAAGAGCACCTTCA GTCGGTAGCGCATTCGCCGATGACGCCCATGCTTCTGAGACTGA TGGGCGGTCGACCTGCTCCATGATGGTGTGGCGTAGGCGTCGGG CCGTCCGCACTTGAGTCCGAATAAGCCAGGCCAAT

Table 3. Result of blastn similarity search.

Sl. No	Primer	Organism	Crops showed similarity	Gene Identity Number (GI. No.)	Accession No.
1.	Af and Ar	orchid	cDNA 5', mRNA of flower bud of *Phalaenopsis violacea*	110663973	CK858452.1
			cDNA 5', mRNA of Lambda ZapII cDNA Library *Phalaenopsis equestris*	109152803	CB034051.1
2.	Bf and Br	orchid	cDNA clone 5', mRNA of flower bud of *Phalaenopsis violacea*	110663423	CK857808.1
			cDNA clone 5', mRNA of flower bud of *Phalaenopsis equestris*	109154466	CK855950.1
3	Df and Dr	orchid	cDNA 5', mRNA of *Ipomoea batatas*	114781594	EE878220.1

Table 4. Result of blastx similarity search

Sl. No	Primer	Organism	Crops showed similarity	Gene Identity Number (GI. No.)	Accession No.
1.	Af and Ar	orchid	*Aerangis kirkii* maturase K protein	71913027	AAZ53016.1
2.	Bf and Br	orchid	*Serapias lingua* LFY-like protein OrcLFY	27544595	DBJ BAC55082.1
3	Df and Dr	orchid	*Oryza sativa* retrotransposon protein, putative, unclassified (japonica cultivar-group)	110288992	GB ABB47352.2

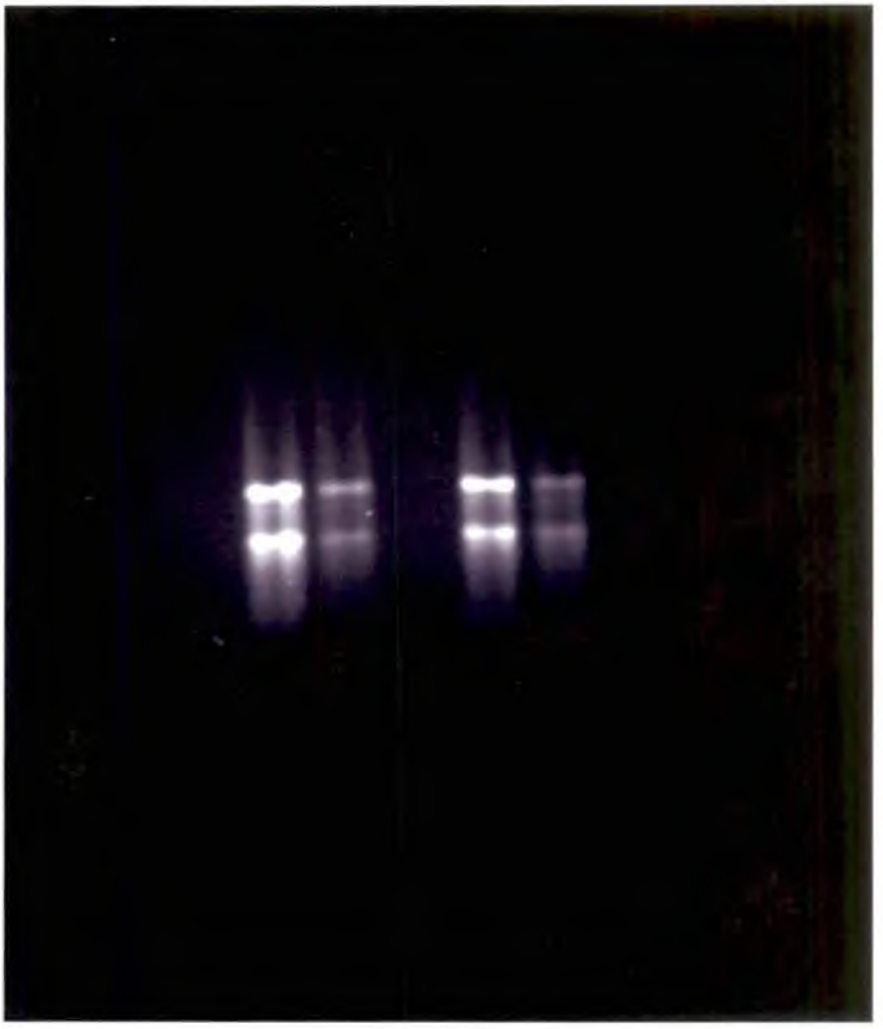

Plate : 3 Total RNA isolated

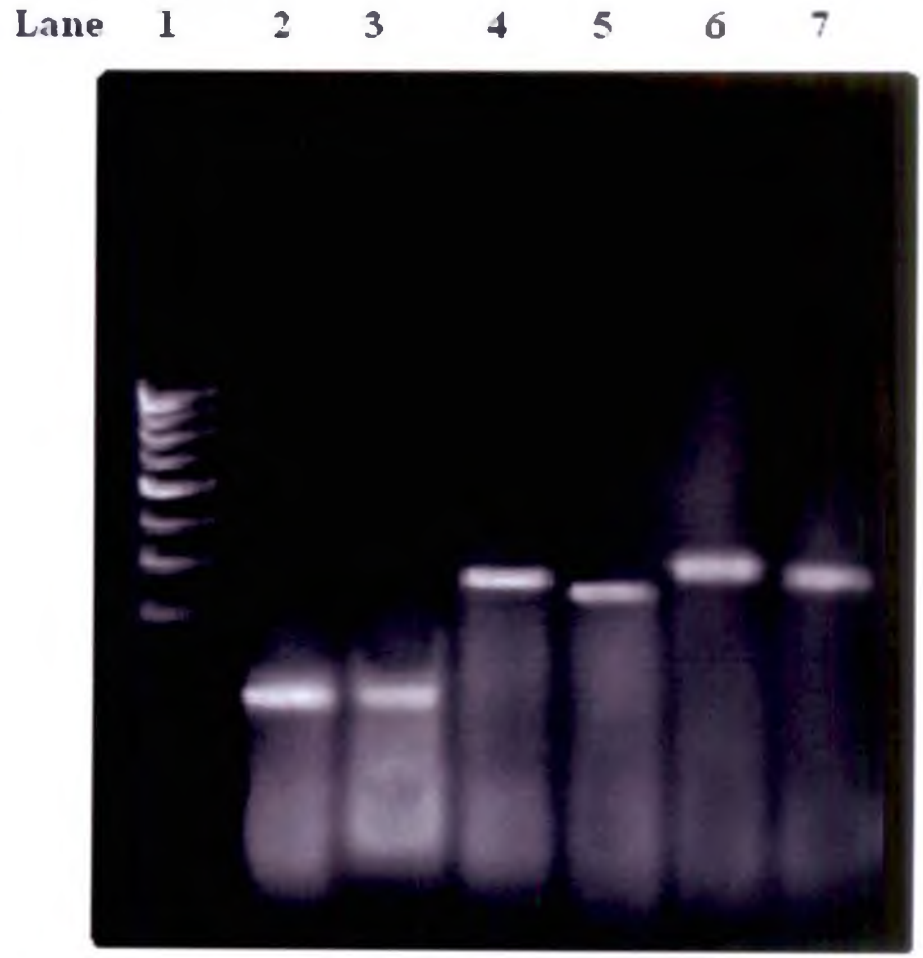

Plate 4: cDNA amplified using chalcone synthase specific primer in orchid

Lane 1 : 100 bp DNA marker

Lane 2 & 3 : cDNA amplified with primers Df and Dr

Lane 4 & 5 : cDNA amplified with primers Af and Ar

Lane 6 & 7 : cDNA amplified with primers Bf and Br

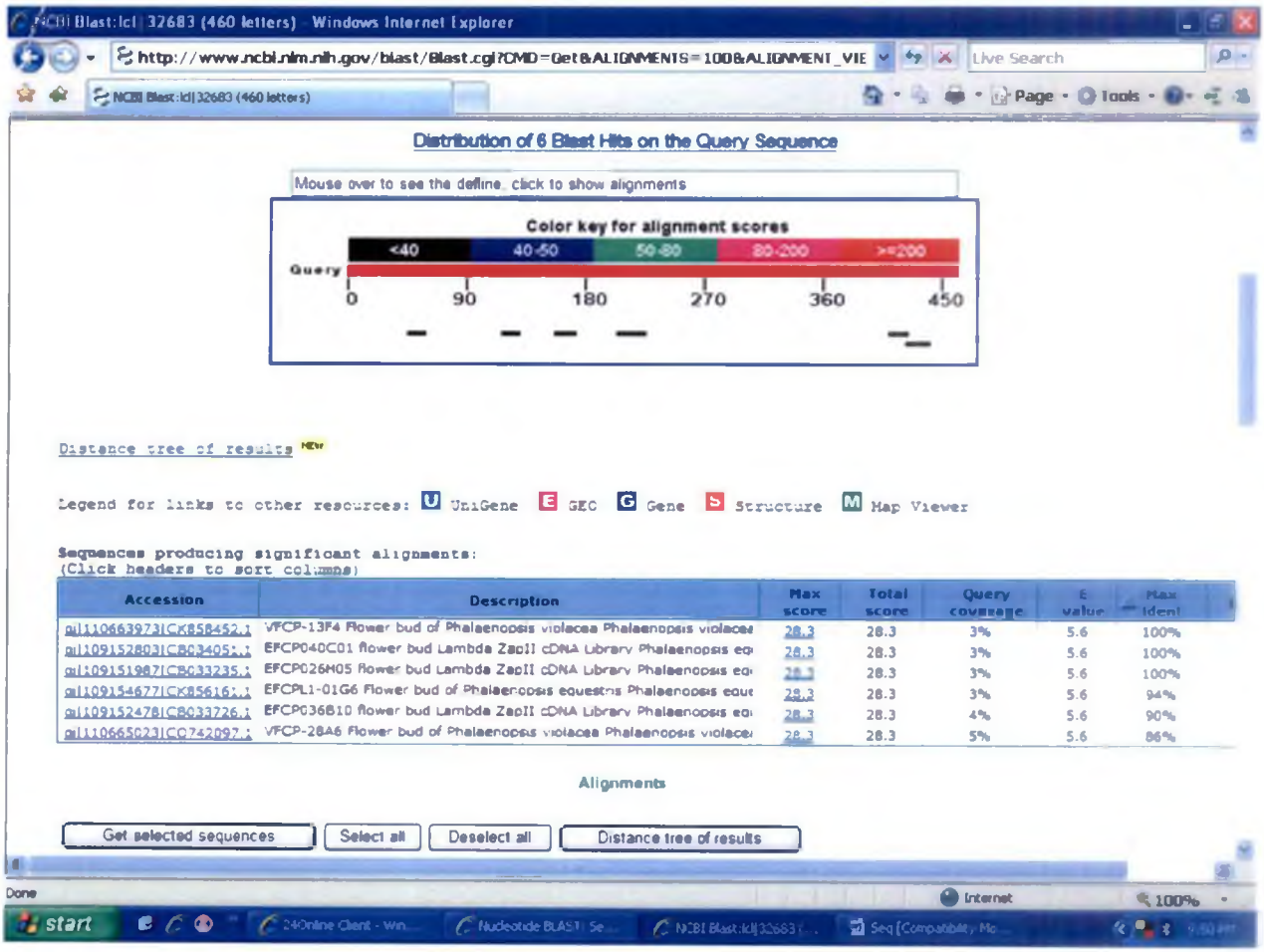

Fig. 2 BLASTN result for cDNA from the flower buds of *Dendrobium* Sonia 17 with primer Af and Ar.

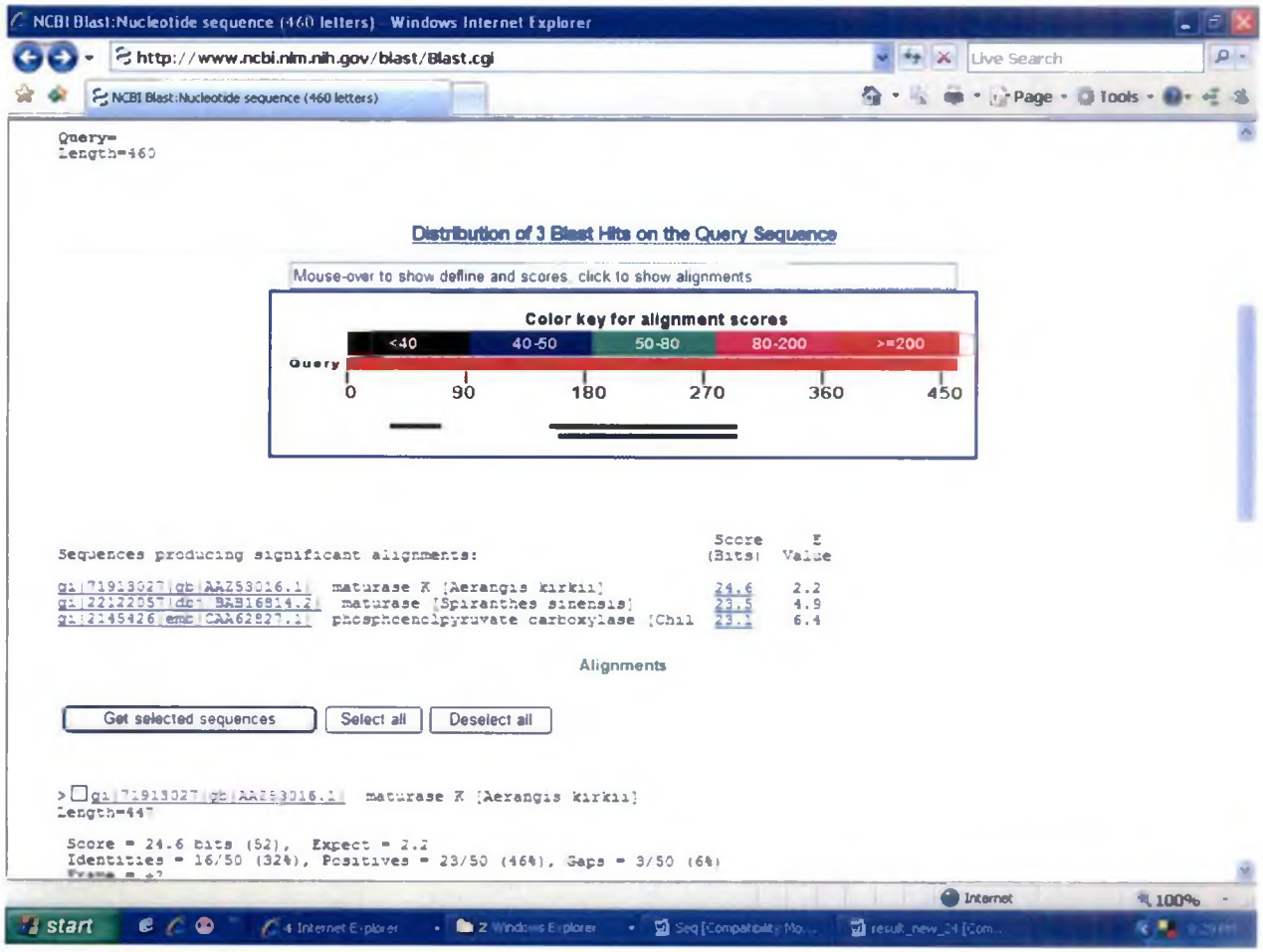

Fig. 3 BLASTX result for cDNA from the flower buds of *Dendrobium* Sonia 17 with primer Af and Ar.

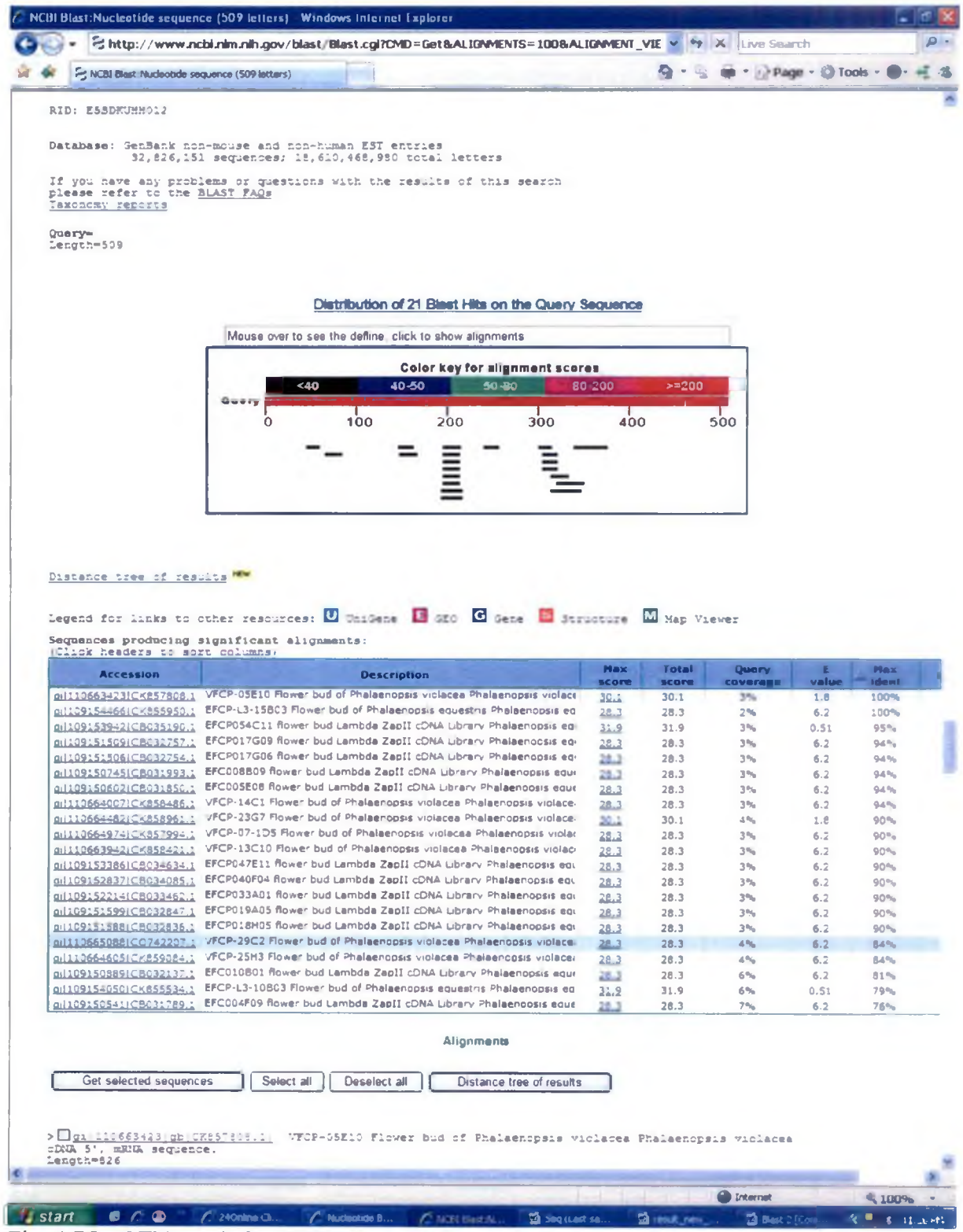

Fig. 4 BLASTN result for cDNA from the flower buds of *Dendrobium* Sonia 17 with primer Bf and Br.

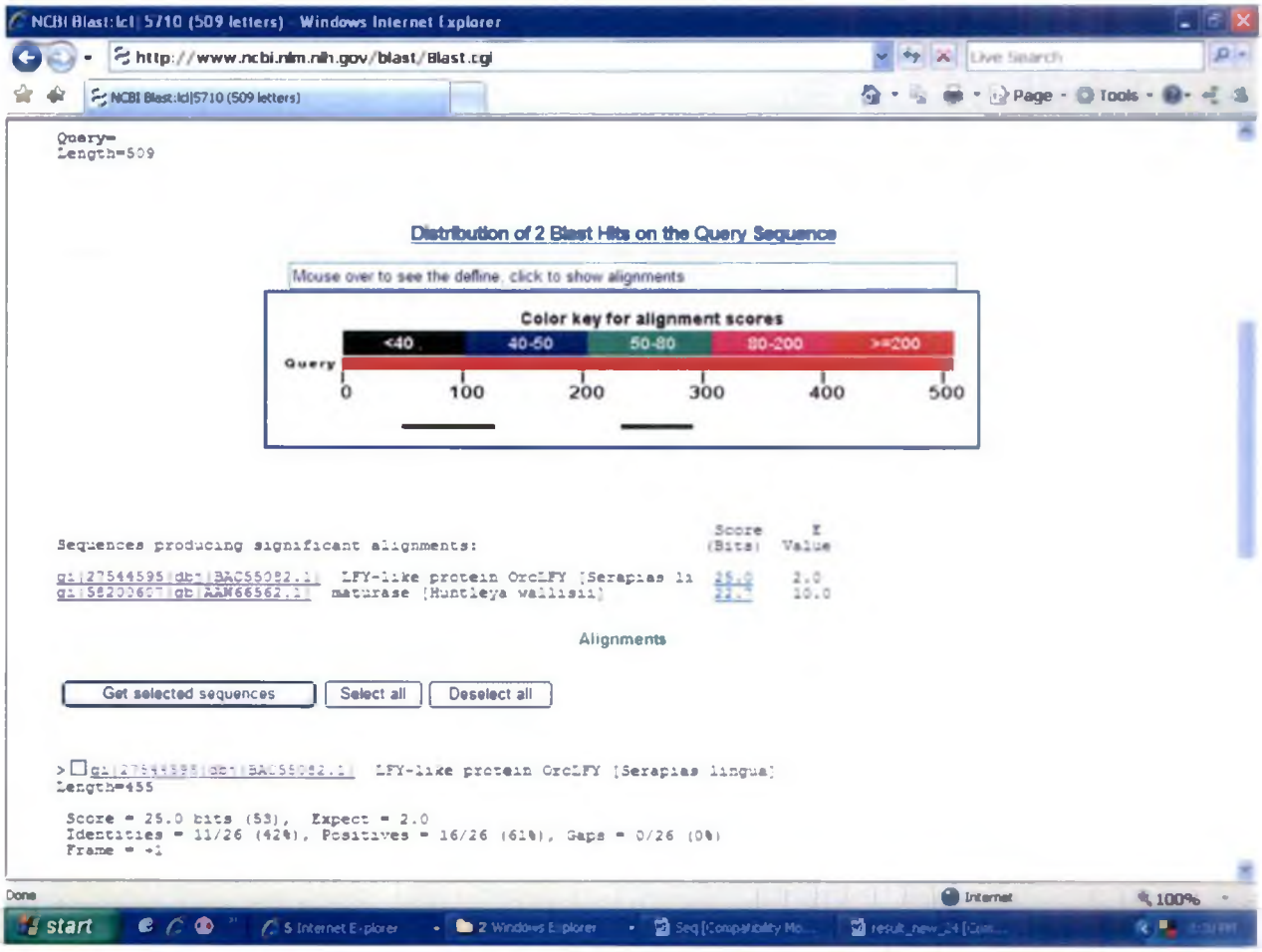

Fig. 5 BLASTX result for cDNA from the flower buds of *Dendrobium* Sonia 17 with primer Bf and Br.

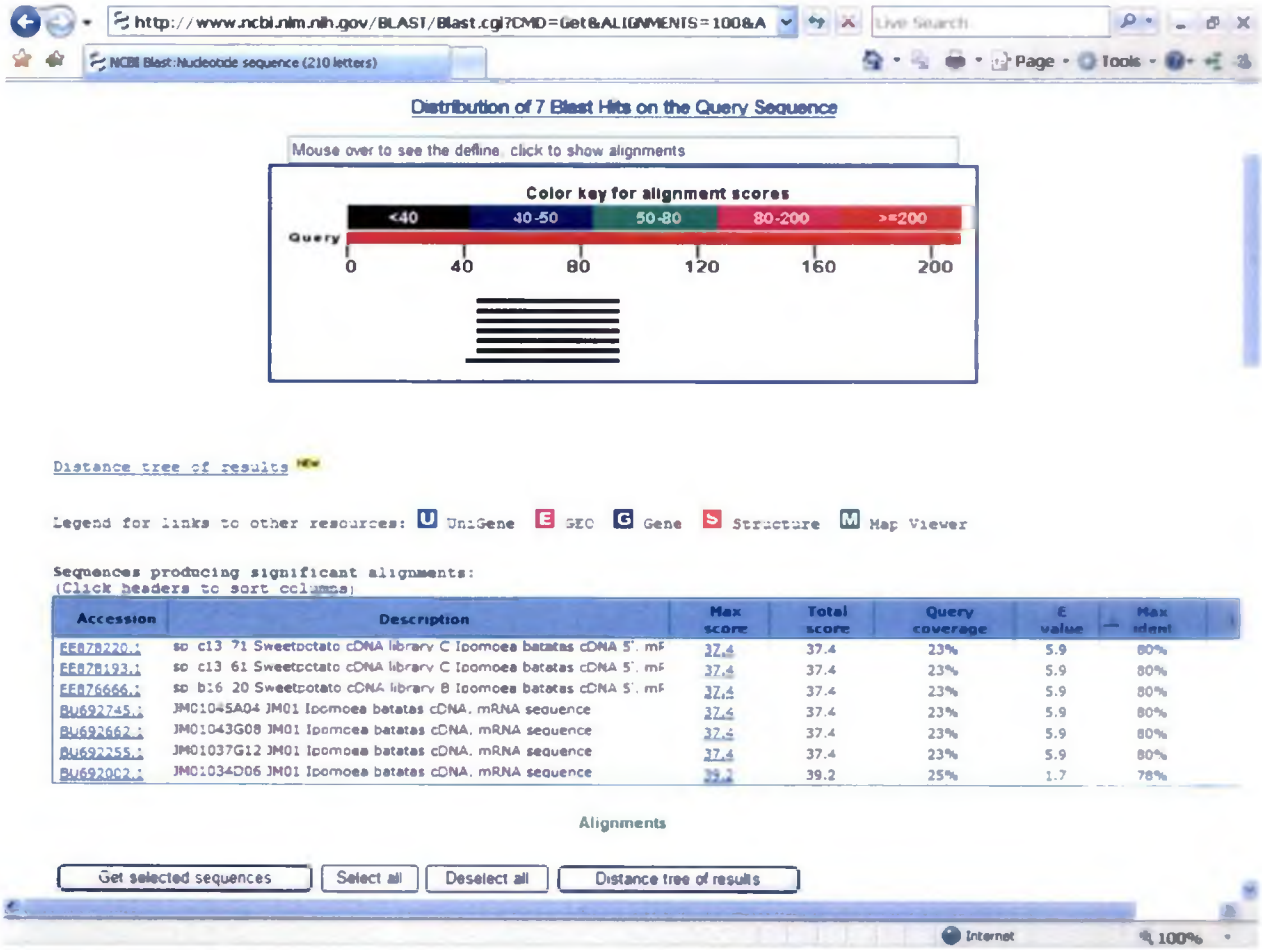

Fig. 6 BLASTN result for cDNA from the flower buds of *Dendrobium* Sonia 17 with primer Df and Dr.

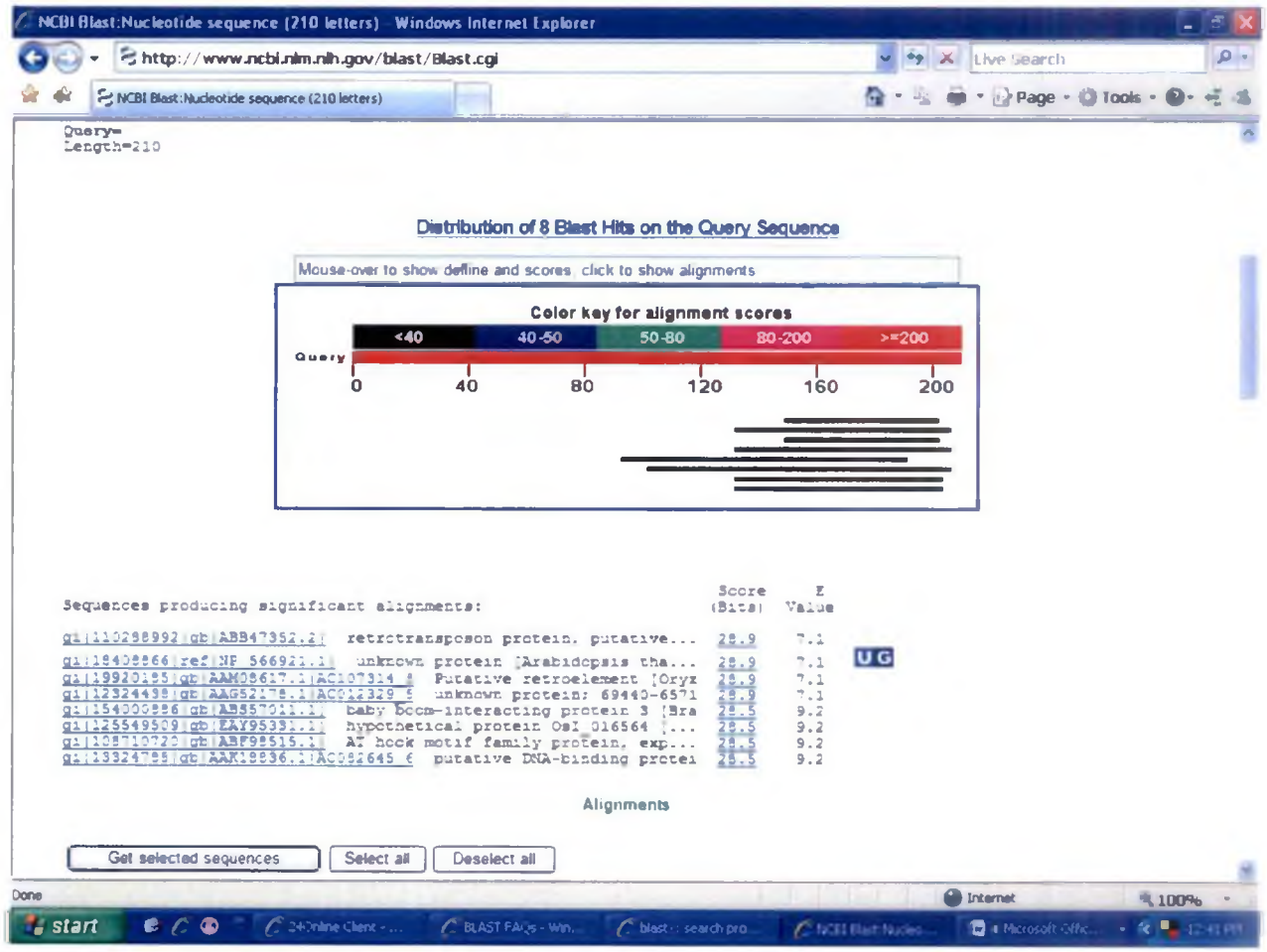

Fig. 7 BLASTX result for cDNA from the flower buds of *Dendrobium* Sonia 17 with primer Df and Dr.

5. Discussion

5. DISCUSSION

Orchids are one of the important cut flower crops. The Global demand for orchids is on the increase. *Dendrobium* is one of the important commercially grown orchid variety currently enjoying high popularity among the farmers in Kerala. Market value of orchid flowers is mainly determined by the floral colouration. Flower colour is influenced by the presence of anthocyanin biosynthetic pathway operating in these crops. Improved and novel flowered varieties will fetch high price in the market compared to the traditional ones. This is possible only by modifying the biosynthetic pathway. This is already been practised in blue/violet carnations and roses and such flowers are on the market also. Not much work has been done on this aspect in orchids.

The flower colour of orchid is due to the presence of anthocyanin. The anthocyanin biosynthetic pathway is very complex with large number of structural and regulatory genes. Any alteration in these genes can affect pigmentation (Jaakola, 2003). The point of regulation of the enzymes cannot be generalized. Recent studies by Quattrachio et al. (1998) indicated that the regulatory genes of anthocyanin pathway are conserved across species and they also suggested that divergent evolution of flavonoid biosynthesis gene promoters is responsible for the species specific difference in regulatory networks.

Factors affecting flower colour are primarily genetically determined. In addition, environmental factors such as nutrients, temperature and light conditions can have an effect on flavonoid composition (Jaakola, 2003).

Three key enzymes *viz;* chalcone synthase (CHS), dihydroflavonol 4- reductase (DFR), and UDP: glucose flavonoid 3-oxy-glucosyl transferase (UFGT) are involved in anthocyanin biosynthetic pathway. Out of which chalcone synthase is the specific enzyme involved. The research was focused on isolation and characterization of this chalcone synthase gene using RT- PCR.

Primer designing was the first step undertaken. Heterologous primers were designed for the specific amplification of chalcone synthase based on the gene sequence of other crops. Primers (Af and Ar) and (Bf and Br) were designed based on the conserved region of the gene *chs* in rice and strawberry respectively. Since the above primers failed to produce expected similarity, primers (Cf and Cr) and (Df and Dr) were designed based on the conserved region of the gene in *Orchid.* One reported set of primer (Ef and Er) were selected from the published work of *chs* gne in *Phalaenopsis* orchid (Ying ying han et al., 2005). Primer3 software was used for this.

Manually designed primers were analyzed using the oligonucleotide properties calculation programme to find out the hair pin formation, 3'complementarity and self annealing property. BLASTN analysis was also done to find out the binding specificity of the primers.

Four protocols were tried for the isolation of total RNA from the floral tissues of orchid. The major problem associated with the RNA isolation was that the RNA obtained was very hard and difficult to dissolve in sterile water. It may be due to the low purity of RNA. Another problem associated with the isolation was the interference of large quantities polysaccharides and polyphenolic compounds. Interference of polyphenolic compounds was overcome by the addition of polyvinyl pyrrolidone (PVP) in the extraction buffer.

The successful method followed for the isolation of good quality RNA was the method developed by Schneitz (2000) which utilizes hot phenol maintained at 80°C for isolating RNA. Orchid floral tissues are deeply coloured, and Pawlowski et al. (1994) reported that the procedure should involve enough washing steps for the complete removal of the coloured residues, otherwise these bind to RNA to form insoluble complexes which may reduce the yield of RNA. So problem was overcome by additional washing steps with ethanol after the first precipitation of RNA with lithium chloride.

The total RNA isolated was gel electrophoresed on 1.4% agarose gel showed two clear bands corresponding to 18S rRNA and 28S rRNA indicating that the isolated RNA was totally intact. The intensity of 28S rRNA was double the intensity of 18S rRNA.

Reverse transcription-PCR was carried out for cDNA amplification. The thermal cyclic reaction used for the designed primers was the same except annealing temperature which was changed with the Tm of primers. The reaction included denaturation at 95°C for two minutes followed by 30 cycles of denaturation at 94°C for 60 seconds, annealing for 52 seconds and extension at 72°C for two minutes. Final extension at 72 °C for seven minutes was given. The PCR conditions for the reported primer set was 94°C for five minutes, 94°C for 60 seconds, 55°C for 60 seconds and 72°C for two minutes with 35 cycles. Final extension at 72 °C for ten minutes was given. During the reaction non specific amplification was observed initially. This problem was solved by decreasing primer concentration and increasing annealing temperature.

After PCR, amplified products were gel electrophoresed on 1.4 per cent low melting agarose gel in 1X TAE buffer at 35 V for five hours. In the case of non specific amplification, bands of the approximate expected sizes were excised from the gel and gel elution was carried out.The purified cDNAs were sequenced.

The amplified product obtained using the primers Af and Ar was 460 bp long and showed maximum similarity to the cDNA clone 5', mRNA sequence of flower bud of *Phalaenopsis violacea* and flower bud of *Phalaenopsis equestris* Lambda ZapII cDNA Library in the BLASTN similarity search. BLASTX analysis of the sequence showed similarity to maturase K protein of *Aerangis kirki.*

Amplified region with primers Bf and Br was sequenced with forward and reverse primer and found to have a length of 509 bp long and showed maximum similarity to the cDNA clone 5', mRNA of flower bud of *Phalaenopsis violacea* and flower bud of *Phalaenopsis equestris* sequence in the BLASTN similarity search. BLASTX analysis of the sequence showed similarity to LFY-like protein of *Serapias lingua.*

The amplified product obtained using the primers Df and Dr was 210 bp long and showed maximum similarity to cDNA 5', mRNA sequence of *Ipomoea batatas* in the BLASTN similarity search. BLASTX analysis of the sequence showed similarity to retrotransposon protein of *Oryza sativa* (japonica cultivar-group).

This work was an initial step towards isolation and characterization of chalcone synthase gene in *Dendrobium* variety Sonia 17. Since the details regarding primer sequence of the chalcone synthase in orchid is not available, primer designing was needed utilizing the gene sequence of chalcone synthase in other crops. The sequences obtained from the study shows similarity with the genes involved in the biosynthetic pathway of *Phalaenopsis* orchid flower fragrance and not with the gene responsible for flower pigmentation. The PCR reactions gave good results but the sequencing result did not give satisfactory result on doing the BLAST search. For the better understanding, detailed studies are needed to be conducted in orchid. Instead of heterologous primers degenerate primers can be used for gene amplification. Methods like RACE and Real Time PCR may be helpful in isolation of full length cDNA and analyzing the difference in the expression of genes involved in anthocyanin synthesis.

6. Summary

6. SUMMARY

The study on isolation and characterization of cDNA encoding chalcone synthase gene from flower buds of orchid *Dendrobium* variety Sonia 17 was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2004 to 2006. The objective of the study was to design primers and sequence the key enzyme viz., chalcone synthase involved in the anthocyanin biosynthetic pathway. Immature flower buds of orchid were subjected to analysis. The salient findings of the study are summarized below.

RT-PCR based approach was used in this study. Since, the details of the reported chalcone synthase primer in *Dendrobium* was not available, heterologous primers were designed based on the gene sequence from rice, strawberry and orchid. The length of the designed primers ranged from 21 nt – 36 nt and Tm from 50.7°C to 65.2°C. Annealing temperature was changed based on the Tm of primers. One reported primer from *Phalaenopsis* orchid was also included in the study.

Extraction of RNA from flower buds was extremely laborious due to the presence of secondary metabolites and mucilaginous nature of the plant. Extraction protocol utilizing hot phenol maintained at 80°C followed by lithium chloride precipitation was the best method for isolation of RNA from orchid floral tissue. Quality of RNA obtained could be improved by an additional washing with 70% ethanol.

The BLASTN search of the cDNA of orchid, amplified using chalcone synthase specific primer Af and Ar, showed significant similarity with cDNA clone 5', mRNA sequence of flower bud of *Phalaenopsis violacea* and flower bud of *Phalaenopsis equestris* Lambda ZapII cDNA Library in the BLASTN similarity search. BLASTX analysis of the sequence showed similarity to maturase K protein of *Aerangis kirki*.

The primers Bf and Br showed maximum similarity with cDNA clone 5', mRNA sequence of the flower bud of *Phalaenopsis violacea* and flower bud of *Phalaenopsis equestris* in the BLASTN similarity search. BLASTX analysis of the sequence showed similarity to LFY-like protein of *Serapias lingua.*

The amplified product obtained using the primers Df and Dr was 210 bp long and showed maximum similarity to cDNA 5', mRNA sequence of *Ipomoea batatas* in the BLASTN similarity search. BLASTX analysis of the sequence showed similarity to retrotransposon protein of *Oryza sativa* (japonica cultivar-group).

This work was an initial step towards isolation and characterization of chalcone synthase gene in *Dendrobium* variety Sonia 17. For the better results, detailed studies are needed to be conducted in orchid with different primers in varying conditions.

7. References

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http://www.basic.northwestern.edu/biotools/oligocalc.html

http://www.sokker.wi.mit.edu.primer3/

APPENDIX I

RNA Extraction buffer (pH 8)

LiCl	0.1M
EDTA	0.01M
SDS	1.0%
Tris	1M (pH 9.0)
PVP	1.5%
BME	2.0%

APPENDIX I1

50X TAE buffer (pH 8.0)

Tris basel	242.0 g
EDTA (0.5M)	100 ml
Glacial acetic acid	57.1 ml

Make up the volume to 1000ml

ISOLATION AND CHARACTERIZATION OF cDNA ENCODING CHALCONE SYNTHASE FROM FLOWER BUDS OF ORCHID *Dendrobium* VARIETY SONIA 17.

ANJANA, G.R

Abstract of the
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Department of Plant Biotechnology
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM 695522

ABSTRACT

The study entitled "Isolation and characterization of cDNA encoding chalcone synthase gene from the flower buds of orchid *Dendrobium* variety Sonia 17" was conducted at the Department of Plant Biotechnology, Vellayani, Thiruvananthapuram during the period from 2005 to 2007 with an objective of studying the isolation and characterization of cDNA encoding chalcone synthase gene involved in anthocyanin pigmentation in orchid flower buds.

. Heterologous forward and reverse primers were designed based on the gene sequences of *Oryza sativa, Fragaria ananasa* and *Phalaneopsis* orchid using primer3 software. Total RNA was isolated from immature floral tissues using hot phenol method which gave an yield of 80 - 200 $\mu g\ g^{-1}$ of the tissue and a A_{260}/A_{280} ratio ranging between 1.6 –2.0. Messenger RNA was purified from the total RNA using the mRNA purification kit from GENEI (Bangalore). Reverse transcription-polymerase chain reaction was carried out to study the expression of gene. The RT-PCR amplified products representing chalcone synthase (CHS) gene was eluted and purified. The product was sequenced and studied the similarity of the same using homology search. All sequenced regions were subjected to BLASTN and BLASTX similarity search.

Rice chalcone synthase specific primer produced an amplified sequence of 460 bp long and showed maximum similarity to the cDNA clone 5', mRNA sequence of flower bud of *Phalaenopsis violacea* and flower bud of *Phalaenopsis equestris* Lambda ZapII cDNA Library in BLASTN similarity search.BLASTX analysis of the sequence showed similarity to maturase K protein of *Aerangis kirki.*

The cDNA amplified with strawberry *chs* specific primer showed maximum similarity to the cDNA clone 5', mRNA sequence of *Phalaenopsis violacea* flower bud

and flower bud of *Phalaenopsis equestris* in the BLASTN similarity search. BLASTX analysis of the sequence showed similarity to LFY-like protein of *Serapias lingua.*

The results of the nucleotide to nucleotide search (BLASTN) of the cDNA of orchid, amplified using chalcone synthase specific primer from orchid showed similarity to cDNA 5', mRNA sequence of *Ipomoea batatas* in the BLASTN similarity search. BLASTX analysis of the sequence showed similarity to retrotransposon protein of *Oryza sativa* (japonica cultivar-group).

The result of the sequences obtained from the study shows similarity with the genes involved in the biosynthetic pathway of *Phalaenopsis* orchid flower fragrance.