DIFFERENTIAL EXPRESSION OF GENES INVOLVED IN ANTHOCYANIN PIGMENTATION IN RED BANANA AND GREEN-RED CLONES

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DECLARATION

I hereby declare that this thesis entitled "**Differential expression of genes involved in anthocyanin pigmentation in Red banana and Green-red clones**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that this thesis entitled **"Differential expression of genes involved in anthocyanin pigmentation in Red banana and Green-red clones"** is a record of research work done independently by Ms. Rehna Augustine (2003-11-52) 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.

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Dedicated to

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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
CBB	Coomassive brilliant blue
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 Alignment
G	Guanine
IPTG	Isopropyl-beta-thio-galactopyranoside
LB	Luria bertaini broth
LiCl	Lithium chloride
M	Molar
mM	millimolar
MMuLV	Moloney Murine Leukemia virus

mRNA	Messenger RNA
NCBI	National Centre for Biotechnology Information
ng	nanogram
PAGE	Poly acrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PMSF	Phenyl methane sulphonyl fluoride
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
SDS	Sodium dodecyl sulphate
SDH	Shikmate dehydrogenase
T	Thymine
TAE	Tris-acetate EDTA
Taq	*Thermus aquaticus*
TE	Tris-HCl –EDTA
TEMED	N, N, N', N' Tetramethyl ethylenediamine
Tris	Tris (hydroxy methyl) aminomethane
Tris-HCl	Tris (hydroxy methyl) aminomethane hydrochloride
U	Uracil
UFGT	UDP: flavonoid 3-Oxy- glucosyl transferase
X-Gal	5-Bromo-Chloro-3-Indolyl-β-D-Galactopyranoside
µl	microlitre
µg	microgram

Introduction

1. INTRODUCTION

India is the second largest producer of fruits in the world. It ranks first in the production of banana. More than 400 lakh people in the tropics depend on banana as staple food. Being the native place of banana, India harbours wide biodiversity of its kind. It is estimated that more than 300 banana cultivars are available in India. Each cultivar is having its own peculiarity. The banana fruit is an excellent source of energy. The fruits are rich in vitamin B6, vitamin C, potassium, magnesium and fibre. In Kerala, banana is cultivated in an area of 1, 10,480 hectares of land with an annual production of 7, 31,950 tonnes (FIB, 2006).

Red banana is one of the important varieties of Kerala in which the colour is a vital commercial factor. The fruit of this variety are highly priced, due to its peculiar colour and flavour. The fruit contain flavonoids, which have been found to own potent antioxidant and free radical scavenging activities. There is growing evidence from human consumption studies supporting a protective role of flavonoids in cardiovascular diseases and cancer. However, there is a major problem in the cultivation of Red banana. Often, a transition of red colour to green is observed among a sizeable number of population. Even though the red one is similar to the green in all other aspects, it fetches lower price in the market, as the consumers prefer the former. Not much work has been done in this area to investigate the cause of colour change.

The phenomenon of colour change occurs in all types of soils and climate and even in tissue culture plants. Hence, the change may not be due to the influence of environmental factors. Also there is no convincing evidence as to the chimeric nature of this phenomenon. Dynamic factors affecting the expression of genes involved in anthocyanin pigmentation and accumulation may be the root cause.

The present study was aimed at differentiating the red and green clones based on the expression of anthocyanin pigmentation genes. Out of the several enzymes participating in the complex anthocyanin biosynthesis pathway, three key enzymes *viz.*, chalcone synthase, dihydroflavonol 4- reductase and UDP: glucose 3-oxy-glucosyl transferase, were selected for analysis. Efforts were also made to distinguish both the clones based on the total protein profile and sugar content.

Review of Literature

2. REVIEW OF LITERATURE

Banana is one of the most important commercial fruit crops of the world. India is the largest producer of banana having an annual production of 97.2 million tones from an area of 88.3 lakh hectare. It contributes to 31.7 per cent of total fruit production. In Kerala also banana is the leading fruit crop with a total area of 110,480 hectares (FIB, 2006). Red banana is a leading variety mainly grown in the southern parts of the country. The fruits of this variety are highly priced due to the peculiar colour and flavour. However, the growers of this variety very often face the problem of transition of red colour to green in sizeable number of plants. Even though, the green fruit is similar to the red in all other respects, it fetches low price in the market, as consumers prefer the latter.

First mention of the colour change from red to green was made by Simmonds (1959). He coined the term 'Green-red' to represent green variants. According to him the change is developmental accident rather than mutation. The frequency of change from Red to Green-red is very high and the reverse has never been recorded. Red banana has red pseudostem, petioles and fruit; whereas Green-red has them green. The red colour is due to anthocyanin, a flavonoid compound which is also responsible for the peculiar flavour. The limited colour transition within the variety can be due to the absence, mutation or altered expression of an anthocyanin biosynthetic gene (Helariutta *et al.*, 1993).

2.1 Flavonoids in Plants

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).

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).

Anthocyanins, the largest flavonoid subclass are the main pigments in flowers and fruits which are responsible for the blue (delphinidins), red (anthocyanins) and purple pelargonidins) colouration. Of all plant secondary compounds, anthocyanins have been investigated most extensively in the areas of chemistry, biochemistry and genetics (Jaakola, 2003).

Red banana, which serve as a source of anthocyanins, have been found beneficial for human health.

2.2 Anthocyanin Biosynthesis Pathway

Flavonoids are synthesized *via* the phenyl propanoid pathway. This pathway starts from the aromatic amino acid phenylalanine, which is synthesized by the shikimic acid pathway. Phenylalanine ammonia lyase (PAL) catalyzes the conversion of phenylalanine to cinnamate. The cinnamate 4-hydroxylase (C4H) catalyzes the synthesis of p-

hydroxycinnamate from 15 cinnamate and 4-coumarate CoA ligase (4CL) converts p-coumarate to its coenzyme-A ester, activating it for reaction with malonyl CoA which serve as the substrate for anthocyanin production (Jaakola, 2003).

Detailed studies conducted by Beld *et al.* (1989), Charrier *et al.* (1995) and Bernhardt *et al.* (1998) on the biochemical and molecular aspects of anthocyanin biosynthetic pathway made the understanding of the pathway easier. The anthocyanin 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).

Anthocyanin biosynthesis starts with the condensation of one molecule of 4-coumaryl-CoA and three molecules of malonyl-CoA, yielding naringenin chalcone. This reaction is carried out by the enzyme chalcone synthase (CHS). Chalcone is isomerised to a flavanone by the enzyme chalcone flavanone isomerase (CHI). From these central intermediates, the pathway diverges into several side branches, each resulting in a different class of flavonoids. Flavanone 3- hydroxylase (F3H) catalyzes the stereospecific 3ß-hydroxylation of (2S)-flavanones to dihydroflavonols. For the biosynthesis of anthocyanins, dihydroflavonol reductase (DFR) catalyzes the reduction of dihydroflavonols to flavan-3, 4-diols (leucoanthocyanins), which are converted to anthocyanidins by anthocyanidin synthase (ANS). The formation of glucosides is catalyzed by UDP glucose-flavonoid 3-Oxy-glucosyl transferases (UFGT), which stabilize the anthocyanidins by 3-Oxy-glucosylation (Harborne, 1994; Bohm, 1998).

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

2.3 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

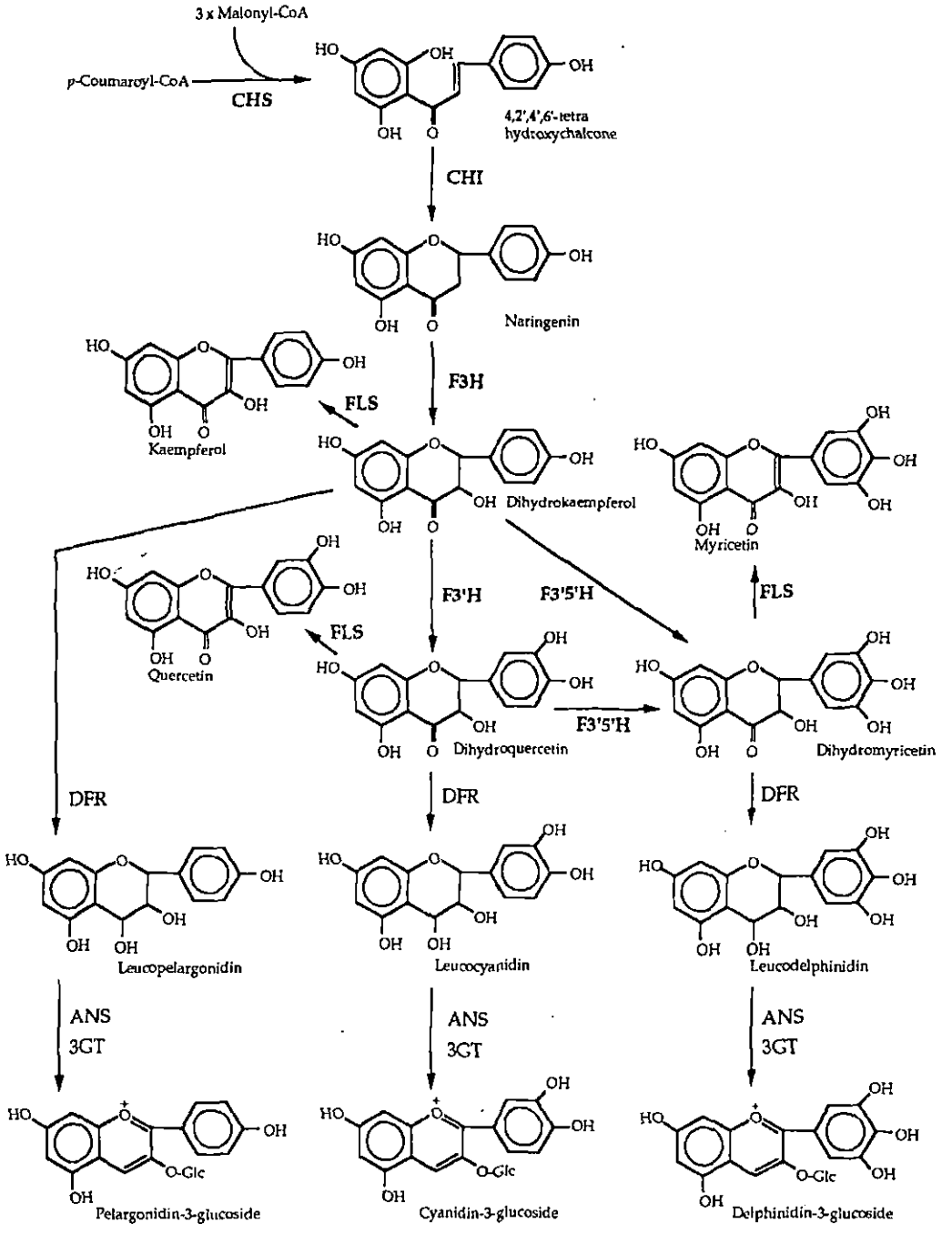


Fig. 1. Anthocyanin and Flavonol biosynthetic pathway

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).

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

2.4 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 etc. can have effect on the final hue of the fruit. Also the temperature and 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).

2.4.1 Light

Studies have shown that light induces anthocyanin accumulation in green tissues and cultured cells *via* the activation of anthocyanin biosynthetic genes (Mol *et al.*, 1996). 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).

2.4.1.1 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).

2.4.1.2 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 was lower in leaves of the red form of *Perilla frutescens*.

2.4.2 Temperature

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

2.4.3 pH

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

2.4.4 Sugars

Flowers and fruits of most plants are heterotrophic and therefore require imported carbohydrates for their development (Halevy, 1987). Increased sucrose concentration enhanced 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).

2.5 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 expression studied (Piero *et al.*, 2005). Major works done on the three key structural enzymes of this complex pathway is described below.

2.5.1 Chalcone Synthase (CHS)

Chalcone synthase (CHS) is a plant-specific polyketide synthase that catalyze the condensation of 4-coumaryl-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).

The first gene isolated from the flavonoid biosynthetic pathway was a chalcone synthase from parsely (*Petroselinum hortens*) by Kruzaler (1983). CHSs have been cloned and sequenced from more than 40 plant species (Abe *et al.*, 2001). Sparvoli *et al.* (1994) isolated CHS gene by screening a cDNA library obtained from mRNA isolated from grapes using snapdragon and maize heterologous probes.

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 chalcone synthase gene, 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.

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

2.5.2 Dihydroflavonol 4- reductase (DFR)

Dihydroflavonol 4- 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).

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

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 was predominantly expressed in early stages of fruit development. The crucial role of this gene in anthocyanin biosynthesis was 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).

2.5.3 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 it has been generally assumed 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 flavonoids 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- glucosyl transferase 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 uncoloured 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.*, (1991) conducted expression analysis in *vitis vinifera* and concluded that UFGT is regulated independently of other genes and the major control point in the pathway is later than that observed in maize, petunia and snapdragon.

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

Expression of specific genes at the level of mRNA can be studied using techniques such as northern blot, dot blot, RNA protection assay, *in situ* hybridization and RT-PCR. 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).

2.6.1 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 and Gelfand (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 preferred Tm 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) at the Whitehead Institute for Biomedical research, U.S.A.

2.6.2 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 reverse transcription-polymerase chain reaction (Liu *et al*, 1998).

To obtain good preparations of eukaryotic mRNA it 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. Martinoff *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, 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.

2.6.3 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 Jakobsen *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.

2.6.4 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. Kolewicz *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.

2.7 Cloning of cDNA

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

Hemsely *et al.* (1989) described a method for cloning PCR products in which the 3'-5' exonuclease activity of bacteriophage T4 DNA polymerase was used to remove extended bases present in the PCR product. The polished DNAs could then be

phosphorylated by T4 polynucleotide kinase and cloned into a blunt ended dephosphorylated vector.

Hinnisdaels *et al.* (1996) used *Pwo* and *Pfu Taq* DNA polymerase for cloning blunt ended DNA fragment. The cloning efficiency was found to be 10-100 folds lower than the efficiencies attained with DNAs equipped with cohesive termini.

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

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

In addition to the basic methods a large variety of techniques to clone amplified fragments have been published, which include ligation independent cloning. (Aslanidis and de Jong., 1990; Haun *et al.*, 1992; Kaluz and Flint., 1994; Schuldiner and Tanner., 1997), UDG cloning (Nisson *et al.*, 1991; Smith *et al.*, 1993), directional cloning using exonuclease III (Kaluz *et al.*, 1992), *in vivo* cloning (Jones and Howard., 1991) and turbo cloning (Boyd., 1993).

2.8 Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used method for analyzing protein mixture qualitatively. This method is based on the separation of proteins according to size. Hence the method can be used to determine the relative molecular mass of protein (Walker, 2002).

Shah *et al*, (2003) used SDS-PAGE to evaluate the biodiversity among four local and two commercial maize varieties. Variations and similarities were observed in protein profile shown by all of them.

Mukhlesur *et al.* (2004) studied genetic diversity in *Brassica* species using SDS-PAGE analysis. They collected 85 different cultivars of *Brassica* and used for the analysis of seed and protein variation by SDS-PAGE to identify the polymorphic genetic markers for evaluation of genetic resources.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Differential expression of genes involved in anthocyanin pigmentation in Red banana and Green-red clones" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram, during the year 2003 to 2005. Details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3.1 MOLECULAR ANALYSIS

3.1.1 Primer Designing

Primers were designed using Primer3 software (Rozen and Skaletsky, 2000). For each of the three genes (Chalcone synthase, Dihydro flavonol 4-reductase, UDP-glucose: flavonoid 3 -oxy- glucosyl transferase), heterologous cDNA sequences from various plant species were downloaded from the National Centre for Biotechnology Information (NCBI) Gen Bank in FASTA format (Table 1). Homologous regions of the genes were identified using Clustal X 1.81 multiple alignment program. The conserved regions were then selected for designing primers with Primer3 (Fig. 2).

The following criteria were specified for designing primers. Primer size 20-22 bases, product size 500-800 bases, primer GC% 50-60, maximum allowable length of a mononucleotide repeat 4, number of consecutive Gs and Cs at the 3' end of both left and right primer 1. The other parameters were set as default.

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

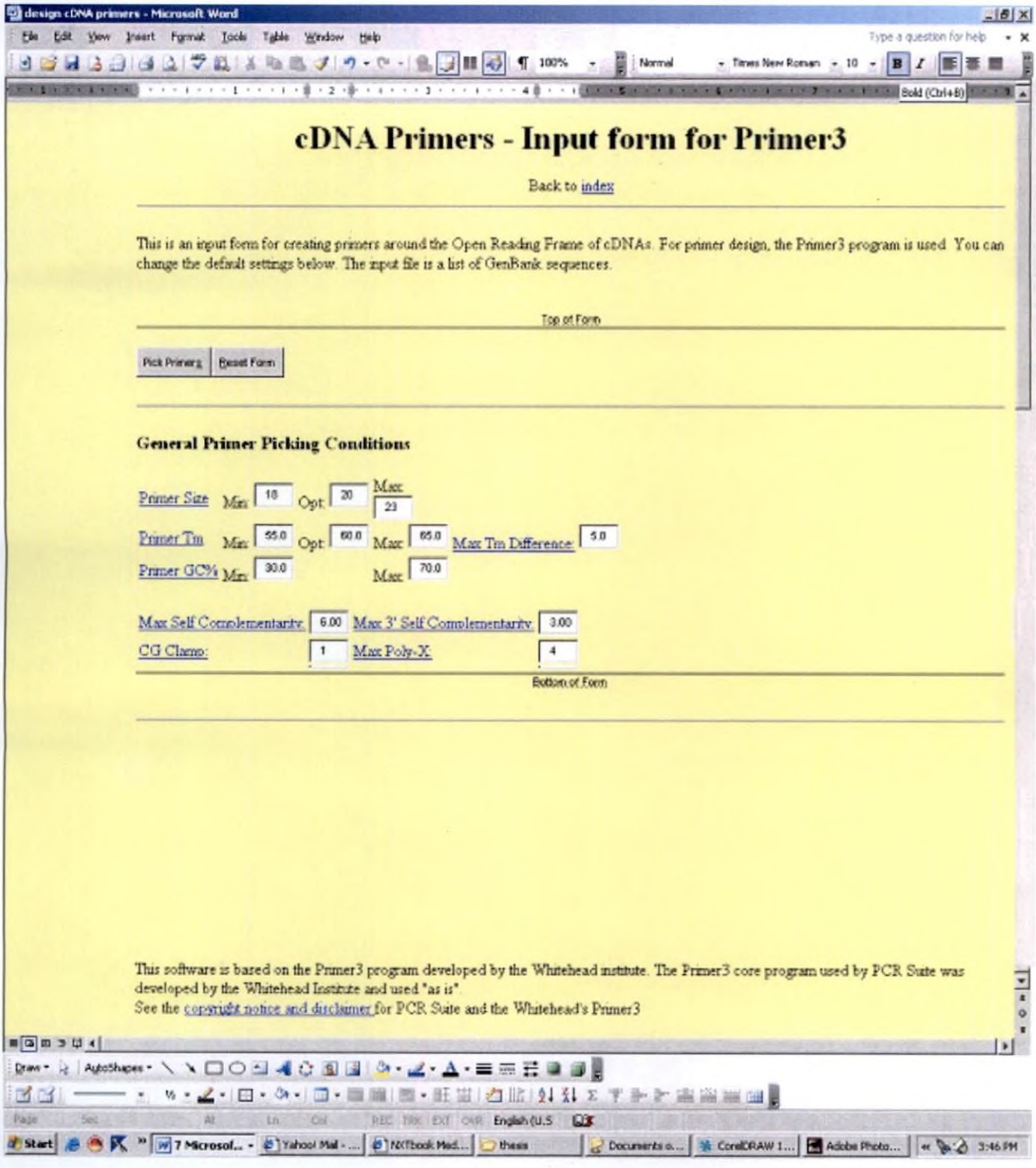


Fig. 2. Input form for Primer3

3.1.2 RT- PCR

3.1.2.1 Plant Material

Messenger RNAs were isolated from both Red banana and Green-red clone using tissues collected from three different stages; tissue culture shoots, unexpanded leaves of field grown plants and immature fruit peel. Tissue culture shoots were purchased from Biotechnology and Model Floriculture Centre, Vegetable and Fruit Promotion Council Keralam, Kazhakkuttam, Kerala. The leaves and immature fruit tissues were collected from the Instructional Farm, College of Agriculture, Vellayani, Thiruvananthapuram.

3.1.2.2 Isolation of Total RNA

All materials used in extraction were free of RNAse contamination. Ribonucleases are very stable enzymes that hydrolyze RNA. They can temporarily be denatured under extreme conditions but it readily renatures. They can survive autoclaving and other standard methods of protein inactivation. To reduce RNase contamination, sterile gloves were worn during the entire course of work. Centrifuge tubes, micro tips, mortar, pestle, spatula and glass wares were treated with 0.1 per cent diethyl pyro carbonate (DEPC), a strong inhibitor of RNases. For this 1 ml of DEPC was added to one litre of distilled water and mixed well. The lab ware 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. After autoclaving the equipment were baked for ten hours at 60°C.

Non disposable plastic wares were rinsed in chloroform, followed by DEPC treated, autoclaved water, to inhibit RNases. Water and solutions were also treated with 0.1 per cent DEPC and autoclaved prior to use. Solutions containing Tris were prepared

in DEPC treated autoclaved water. Electrophoresis tanks were soaked in three percent hydrogen peroxide (v/v) for 10 -15 minutes and then rinsed with RNase free water.

Seven standard protocols were tried for the isolation of total RNA from banana. The first method tried was using mRNA purification kit of GENEI (Bangalore). In the second method guanidine thiocyanate was used as protein denaturant. Extraction was also carried out with CTAB buffer. Isolation of RNA with extraction buffer containing ammonium thiocyanate was also tried. RNA isolation method developed at Rubber Research Institute of India which utilizes 3M lithium chloride for precipitation of RNA from lateciferous tissues was also used for banana. Extraction of total RNA with phenol chloroform followed by ethanol precipitation was also checked. The method developed by Schneitz (2000) was used for the isolation of total RNA from banana tissues based on the quality of RNA isolated.

Plant tissue (one gram) was ground into fine powder in liquid nitrogen in a pre-treated mortar and pestle (Appendix I). The 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. The contents were then vortexed to avoid isolation of genomic DNA. The mixture was centrifuged at 10000 rpm for 15 minutes in a refrigerated centrifuge at 4°C. Supernatant was then transferred to a new tube to which an equal volume of chloroform was added. The tubes were shaken at room temperature for 30 minutes in an incubator shaker. After that the mixture was centrifuged at 12,000 rpm for 20 minutes. 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. The tubes were incubated overnight at -20°C to precipitate RNA. The precipitated RNA was pelleted at 10,000 rpm for 15 minutes in a refrigerated centrifuge. Pellet was dissolved in 0.5ml 2 M LiCl and was centrifuged for five minutes at 10,000 rpm. Recovered RNA was then washed twice with 70 per cent ethanol. To purify further, the pellet was again washed with absolute ethanol and air dried for 15 to 30 minutes. Finally, the dried pellet was dissolved in 50 µl RNase free water and stored at -80°C.

3.1.2.3 Quantification of RNA

UV Spectroscopy was used for assessing the concentration of RNA. 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.

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.

3.1.2.4 Agarose Gel Electrophoresis

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. The gel was stained with ethidium bromide and analyzed using gel documentation system (BIO-RAD).

3.1.2.5 Purification of mRNA from Total RNA

The total RNA was added to the vial of oligo $d(T)_{25}$ silica (mRNA purification kit KT 80, Bangalore GENEI) 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.

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. The step was repeated twice.

To elute mRNA 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.

3.1.2.6 RT - PCR

The isolation of intact 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. RT-PCR was carried out using the RT-PCR kit (Bangalore GENEI, KT- 24) in a Programmable Thermal Controller PTC-100 (MJ Research).

3.1.2.6.1 Materials

Good quality mRNA, oligo $d(T)_{18}$ primer, RNase inhibitor, 5X RT buffer, 30m M dNTP mix, AMV reverse transcriptase, 0.1M DTT, 10X PCR buffer, *Taq* DNA polymerase, gene specific primers.

3.1.2.6.2 First Strand Synthesis

The cDNA was prepared from 5ul of mRNA, which was reverse transcribed by AMV reverse transcriptase (Bangalore GENEI) from an oligo $d(T)_{18}$ primer using standard methods in a reaction volume of 20 µl.

1-5 µl of mRNA sample (10 -100ng) was added to a sterile PCR tube. Nuclease free water was added to the tube to bring the volume to 9µl. 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:

Item	Volume (µl)
RNase inhibitor	1.0
0.1 M DTT	1.0
RT buffer (5x)	4.0
30mM dNTP mix	2.0
Reverse Transcriptase	0.5
Sterile water	1.5
Total	25.0

The solution was mixed well and incubated first at 42°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.

3.1.2.6.3 PCR Amplification

The cDNA was amplified using gene specific primers. 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	2.0
10x PCR buffer	1.0
30mM dNTP mix	1.0
Forward primer (100ng/µl)	1.0
Reverse primer (100ng/µl)	1.0
Taq DNA polymerase	0.5
Sterile water	14.5
Total	25.0

Gene specific primers 5' GAC AAG TCG CAG ATC AGG AAG 3' and 5' CTA CCA ACA CAC GGG AAA AGG 3', 5' GCG AAT CCA ACA CAA GCA C 3' and 5' AGC GTG TAC CTG AAC CTG AAC 3' and 5' GAC AAG TCG CAG ATC AGG AAG 3' were used to amplify chalcone synthase, Dihydroflavonol 4-reductase and UDP: flavonod 3- oxy glucosyl transferase, respectively. The PCR conditions were 95°C for 2 minutes 94°C for 60 seconds, 53°C for 40 seconds and 72°C for 80 seconds with 35cycles. Final extension at 72 °C for five minutes was given.

Control reactions containing only forward or reverse member of a primer pair to distinguish the target product from non target products and primer dimer formation were also carried out.

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. 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, again PCR amplified and purified for cloning as described below.

Excised cDNA containing low melting agarose gel slice was placed in a 1.5 ml micro centrifuge tube. The slice was melted by incubating the tube at 65°C. To the gel

mix one volume of TE saturated phenol was added and 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.

3.1.3 Cloning

The specific PCR products were cloned using TA cloning kit (Invitrogen, Inc., USA). For this the PCR products with 3' overhangs were first ligated into the vector, followed by transforming competent *E. coli*. The transformants were analyzed by blue white screening and correct recombinants were selected to isolate plasmid DNA. The correct recombinant plasmid was then purified further for sequencing.

3.1.3.1. Amplifying the Products; Adding 3' Overhangs

PCR was carried out for adding 3' overhangs to cDNA products. The reaction was set in 25 µl volume containing:

Item	Volume (µl)
DNA template	2.0
10 x PCR buffer	2.5
50 mM dNTP mix	2.0
Forward primer	0.5
Reverse primer	0.5
Taq polymerase	0.3
Sterile water	17.2
Total volume	25.0

3.1.3.2. Ligation into Vector, pCR II

PCR products with 3' overhangs were ligated into vector. The vector used was pCR II. The volume of PCR product needed for optimum ligation to the vector was determined using the following formula

$$\text{X ng PCR product} = \frac{\text{Y bp PCR product 50 ng pCR II vector}}{\text{Size in bp of the vector: } \sim 3900}$$

Ligation reaction was set as follows:

Item	Volume (µl)
Fresh PCR product	2.5
10 X ligation buffer	1.0
pCR II vector (25 ng / µl)	2.0
T4 DNA ligase (4 Weiss units)	2.0
Sterile water	2.5
Total volume	10.0

The ligation reactions were incubated at 14°C for eight hours. After ligation the tubes were stored at -20°C.

3.1.3.3 Transforming Competent Cells

The competent cells (TOP10F' chemically competent *E. coli*) were obtained from Invitrogen, Inc., USA. Transformation was done immediately after thawing the cells on ce as competent cells are highly temperature sensitive. Additions were mixed by stirring gently with a sterile pipette tip. The cells were kept on ice during all the steps.

The vials containing the ligation reactions were centrifuged briefly and placed on ice. One 50 µl vial of frozen competent cells was used for each transformation. 2µl of each ligation reaction were pipetted out directly into the vial of competent cells and mixed gently by stirring with a pipette tip. The vials were then incubated on ice for 30 minutes. After incubation, heat shock was given to the cells for 30 seconds at 42°C without shaking. Thereafter, the vials were immediately placed on ice. 250 µl of S.O.C medium (Appendix II) was added to each vial and the vials were shaken horizontally at 37°C for one hour in a shaking incubator.

25µl – 200µl from each transformation vial were spread on agar plates containing X- Gal, IPTG and 50µg/ml kanamycin. The plates were prepared by adding 15g/l of agar in to Luria Bertani (L.B.) broth. After autoclaving, the medium was brought to 55°C and 40µl each of 100mM Isopropyl-beta-thio-galactopyranoside (IPTG), 40 mg/ml 5-Bromo-Chloro-3-Indolyl-β-D-Galactopyranoside (X-Gal) and 50 µg /ml kanamycin were added to the medium. The contents were then dispensed in to sterile petri plates. The plates were incubated overnight at 37°C. Next day, the plates were shifted to +4°C to allow proper colour development.

3.1.3.4. Analyzing Transformants

The transformants were analyzed by blue white screening. The correct recombinants were selected to isolate plasmid DNA. The recombinant plasmid was then purified further for sequencing. Plasmids were isolated from the overnight cultures using alkali lysis method modified by Birnboim and Doly (1979) (Appendix III).

Ten white colonies were picked from each plate for plasmid isolation. The colonies were grown overnight in 2 - 5 ml of LB broth containing 50µg /ml kanamycin. 1.5 ml of fresh cultures were poured into 2ml microcentrifuge tube and spun at 10,000 rpm for one minute at 4°C. The supernatant was removed completely without disturbing the pellet. The pellet was resuspended in 100µl ice cold solution I. 200ul of freshly prepared solution II was added to the mixture and contents were mixed well by inverting

the tubes rapidly five times. The tubes were then stored on ice. 150 µl of ice cold solution III was added to the tubes. To dispense the solution III through the viscous bacterial lysate, tubes were vortexed gently in an inverted position for ten seconds and stored on ice. After five minutes the mixture was centrifuged at 10,000 rpm for five minutes at 4°C. The supernatant was then transferred to a fresh tube and the double stranded DNA was precipitated by adding two volumes of absolute ethanol at room temperature. Ten minutes later the tubes were centrifuged at 10,000 rpm for ten minutes at 4°C. The pelleted DNA was then rinsed with 70 per cent ethanol, air dried and dissolved in 50ul TE buffer. The DNA was stored at -20°C.

The purity was checked in one per cent agarose gel in 1X TAE buffer.

3.1.3.4. Long Term Storage

The original colony was streaked on LB plates containing 50µg/ml kanamycin. From the plate single colony was isolated and inoculated into 1 - 2 ml of LB containing 50µg/ml kanamycin. Cultures were grown until stationary phase was reached. 0.85 ml of culture was mixed with 0.15 ml of sterile glycerol, transferred to a cryovial and stored at - 80°C.

3.1.4 Clone 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.

3.1.5 Data Analysis

Similarity searches were carried out in the NCBI database using nucleotide – nucleotide (blastn) and translated query *vs.* protein database (blastx) searches to identify homologous sequences from other species. Multiple sequence alignment of the Red

banana with Green-red clones was also done to reveal the similarity between the two, with respect to each gene under study.

3.2 BIOCHEMICAL ANALYSIS

3.2.1 Total Protein Profile

Total protein profiles of the two clones were compared by carrying out sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), following the procedure developed by Laemmli (1970).

3.2.1.1 Plant Material

Tissues collected at different stages of the plant namely tissue culture shoots, leaves and fruits of field grown plants were taken for analysis.

3.2.1.2 Protein Extraction

One gram of each sample was homogenized in 1ml of extraction buffer (Appendix IV). The homogenate was then centrifuged at 6000 rpm for ten minutes. The supernatant was transferred to a fresh 2ml microcentrifuge tube and was used for analysis.

3.2.1.3 SDS-PAGE

Polyacrylamide gel was prepared as follows (Appendix V). Glass plate sandwich was prepared using clean glass plates and 1mm thick teflon spacers. Spacers were kept in place by applying grease. The assembly was fixed vertically onto a casting unit. Bottom of the assembly was sealed with 1 per cent agar. Appropriate resolving gel mixture (12%) was prepared and poured into the space between the glass plates leaving sufficient space

at the top for stacking gel. The mixture was overlaid with a thin layer of ethanol to accelerate the polymerization process.

After polymerization the overlying solution was poured off and the top portion was filled with stacking gel mixture (5%). Teflon comb (13 well, 1mm thick) was placed over the stacking gel mixture leaving 1 cm distance between the wells and resolving gel. Care was taken to avoid formation of air bubbles in the gel. Ethanol was poured over the surface and the assembly was left undisturbed for 30 minutes. After polymerization, ethanol was removed and the gel was fixed onto the vertical electrophoresis unit (Bangalore GENEI). Reservoir buffer was added to lower and upper tanks. Before adding buffer to upper tank comb was removed carefully and the wells were washed with reservoir buffer.

3.2.2 Total Sugar Estimation

Total sugar in content in the ripe fruits of Red banana and Green-red clone were estimated using phenol sulfuric acid method (Dubois, 1956). Fruit peel, pulp, peel along with pulp were used for estimation.

Samples were prepared by homogenizing 1g of the tissue in 5ml of distilled water. The homogenate was then filtered through two layers of muslin cloth. 0.2ml each of the samples was then pipetted out into boiling tubes. This broth was then evaporated over a Bunsen burner flame. Once the water had evaporated from the solution, 2ml of sterile distilled water was added to the now dry sugars and mixed in a vortex. 1ml of 5 per cent phenol solution was added to this mixture followed by 5.0ml of concentrated sulfuric acid (18 M). The contents were mixed well to distribute the heat created by the exothermic reaction. This mixture was allowed to cool for around 30 minutes. The absorbance to orange colour that developed is then read in a spectrophotometer at 490 nm, against a reagent blank. Glucose was used to prepare standard curve. Total sugar content in the sample was obtained from the standard curve.

Table 1. Details of cDNA sequences used for designing primers

Sl. No	Gene	Accession No.	Source
1	Chalcone synthase	X89859	*Oryza sativa* (indica cultivar-group)
2	Chalcone synthase	X60204	*Zea mays*
3	Chalcone synthase	AY069951	Sorghum bicolor (sorghum)
4	Chalcone synthase	AY997297	Fragaria ananassa
5	Dihydroflavanol 4 - reductase	AY373831	*Triticum aestivum* (bread wheat)
6	Dihydroflavanol 4 – reductase	AB003496	*Oryza sativa* (japonica cultivar-group)
7	Dihydroflavanol 4 – reductase	AY953938	*Oncidium* Gower Ramsey
8	Dihydroflavanol 4 – reductase	AF017451	*Cymbidium* hybrid
9	UDP glucosyl transferase	AB047097	*Vitis vinifera*
10	UDP glucosyl transferase	AB192315	*Ipomoea purpurea* (morning glory)
11	UDP glucosyl transferase	AB013596	*Perilla frutescens*
12	UDP glucosyl transferase	AY663784	*Fragaria ananassa*

Plate 1. Red and Green-red clone in the field

Plate 2. Tissue culture shoots of Red banana

Plate 3. Immature fruits of Red banana and Green-red clone

Plate 4. Fruit bunches of Red banana and Green-red clone

Plate 5. Fruit development stages of Red banana

Plate 6. Fruit development stages of Green-red clone

Results

4. RESULTS

Gene expression analysis was carried out in Red banana and Green-red clones with the objective of differentiating the two clones based on the expression of chalcone synthase, dihydroflavonol 4- reductase and UDP: glucose 3-oxy-glucosyl transferase, the three key enzymes of the anthocyanin pigmentation pathway. The results of the study are presented in this chapter.

4.1 MOLECULAR ANALYSIS

4.1.1 Primer Designing

Gene specific primers for the three candidate genes were designed (Table 2) using the software Primer3. Both forward and reverse primers were designed and more than one primer combinations were obtained for each gene.

The best primer combinations for each of the three genes were 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. Two sets of primers were selected for each gene under study.

For chalcone synthase, primers designed from rice and strawberry sequences were selected. Primers designed using dihydroflavonol 4- reductase sequences obtained from cymbidium and rice were selected for amplifying dihydroflavonol 4- reductase gene. For UDP: glucosyl transferase, primers designed from grapes and strawberry sequences, were selected. The primers were first tested for PCR amplification of genomic DNA of banana. The primers which gave amplification were chosen for RT-PCR.

4.1.2 RNA Isolation

Out of the seven protocols used for the extraction of RNA, the methods utilizing lithium chloride to precipitate RNA were good. The pellet obtained by extracting RNA at room temperature was too hard and difficult to dissolve in RNase free water. The protocol which utilized hot phenol (pH 8) maintained at 80°C for extraction was the most effective for RNA isolation from banana tissues, especially from fruit peel.

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 7). 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 reactions were carried out using the selected primers for gene specific amplification (Plate 8). Primers designed with rice chalcone synthase mRNA sequences gave better amplification of cDNA isolated from Red banana and Green-red clones, compared to that obtained using primers based on strawberry. The cDNA amplified in both Red and Green-red clones with chalcone synthase specific primer showed a single band of approximately 200 bp.

The primer designed from mRNA for dihydroflavonol 4- reductase from rice gave better amplification. Gene specific amplification yielded two bands, approximately 300 bp and 500 bp, from both Red banana and Green-red clones. The band of size 300 bp was reproducible and was eluted from the agarose gel for cloning.

Plate 7

Total RNA isolated at different stages of Red banana and Green-red clone

Lane 1: Tissue culture shoots of Red banana

Lane 2: Tissue culture shoots of Green-red clone

Lane 3: Leaf tissues of Red banana

Lane 4: Leaf tissues of Green-red clone

Lane 5: Fruit peel of Red banana

Lane 6: Fruit peel of Green-red clone

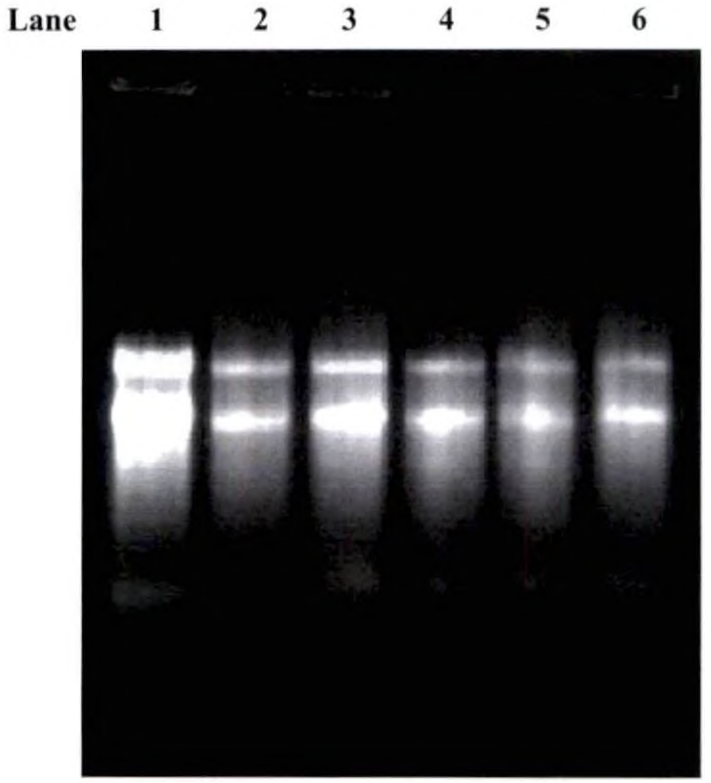


Plate 7. Total RNA isolated at different stages of Red banana and Green-red clone

Plate 8

cDNA amplified using gene specific primers in Red banana and Green-red clone

Lane 1: 100 bp DNA marker

Lane 2: cDNA amplified from Red banana using chalcone synthase specific primer

Lane 3: cDNA amplified from Green-red clone using chalcone synthase specific primer

Lane 4: cDNA amplified from Red banana using dihydroflavonol 4-reductase specific primer

Lane 5: cDNA amplified from Green-red banana using dihydroflavonol 4-reductase specific primer

Lane 6: cDNA amplified from Red banana using UDP: glucose 3-oxy-glucosyl transferase

Lane 7: cDNA amplified from Green-red clone using UDP: glucose 3-oxy-glucosyl transferase

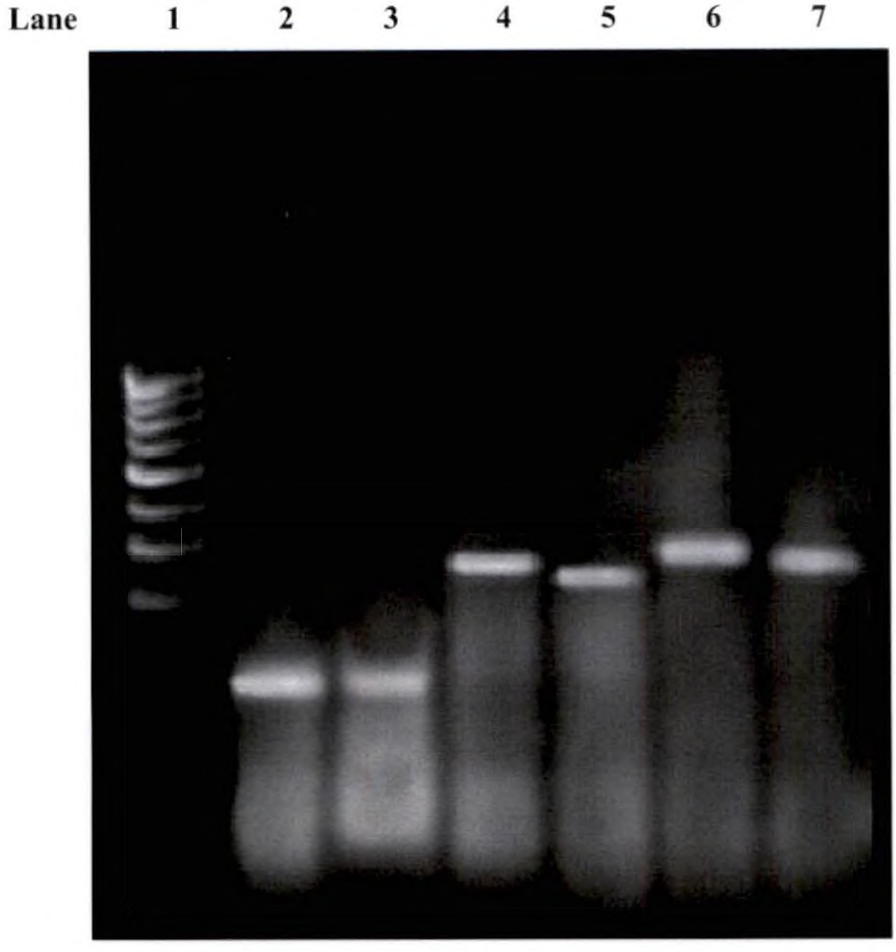


Plate 8. cDNA amplified using gene specific primers in Red banana and Green-red clone

For the amplification of cDNA from Red banana and Green-red clones, the primer designed from mRNA sequence of UDP: glucosyl transferase from grapes gave good amplification. The amplification with UDP: glucosyl transferase yielded two bands of approximately 350 bp and 450 bp. The 400 bp band was faint. Hence, the 350 bp band was cloned.

Primer dimers were visualized in all the cDNA samples during amplification. The reactions were then optimized by lowering the concentration of primers and by increasing the annealing temperature.

4.1.4 Cloning

The amplified product cloned to pCR II vector produced 100 per cent transformants on LB agar plates supplemented with 100mM IPTG, 40 mg/ml X-Gal and 50 µg /ml kanamycin after overnight incubation at 37°C. Transformed colonies appeared small and white. Transformed *E. coli* showed better growth on S. O. C. medium, compared to LB broth.

4.1.5 Sequencing of cDNA Clones

Complementary DNA amplified with chalcone synthase specific primer after sequencing was found to have a size of 183 bp in Red banana. In Green-red also the amplified product was of the same (183 bp). cDNA amplified with dihydroflavonol 4-reductase specific primers showed a fragment of size 354 bp in Red and 325 bp in the Green-red clone. cDNA amplified with UDP: glucose 3-oxy- glucosyl transferase primer in the Red clone was of size 361 bp. In the Green-red clone the product size was 345 bp in length (Table 3).

4.1.6 Similarity search

Characterization of the cDNA clones was done through homology search with blastn (nucleotide with nucleotide search) and blastx (nucleotide to protein) program of NCBI.

4. 1. 6. 1 Chalcone synthase

The cDNA clone which was synthesized from the mRNA of Red banana using chalcone synthase specific primers showed a similarity with *Oryza sativa* (Acc. No. X89859) mRNA for chalcone synthase (Fig. 3). Blastn search of cDNA clone from Green-red clone (Fig. 4) showed similarity with *Medicago truncatula* (Table 4) clone mth2-23o13 (Acc. No. AC14459.8).

The translated query *vs.* protein database (blastx) search pointed out a similarity with the enzyme shikimate 5-dehydrogenase from *Oryza sativa* (japonica cultivar-group, Acc. No. NP_918761) for cDNA clone from Red banana. Blastx search revealed a similar match with a putative 3- dehydroquinate dehydratase/ shikimate 5-dehydrogenase enzyme of *Oryza sativa* (japonica cultivar-group, Acc. No. NP 918759) from cDNA clone obtained from Green-red clone (Table 7).

4. 1. 6. 2 Dihydroflavanol 4 – reductase

The results of the nucleotide to nucleotide search (blastn) of the cDNA clone of Red banana, amplified using dihydroflavonol 4- reductase specific primer, showed no significant similarity with any dihydroflavonol 4- reductases available in the Gen Bank (Fig. 5). The nearest similarity was with *Triticum aestivum* (AY829002) plasma membrane H+-ATPase gene. The cDNA clone of Green–red clone showed similarity

(Fig. 6) with *Physcomitrella patens* (DQ389162) cytokinin receptor 1 (CRE1) gene (Table 5).

The translated query *vs.* protein database search revealed similarity with a hypothetical protein, 5'-partial from *Oryza sativa* ((japonica cultivar-group, Acc. No. AAL79707) for cDNA clone of Red banana. The translated query *vs.* protein database search for similar protein revealed similarity with OSJNBa0053B21.12 from *Oryza sativa* ((japonica cultivar-group, Acc. No. CAE05538) for cDNA from Green-red clone (Table 7).

4. 1. 6. 3 UDP: glucose flavonoid 3-oxy-glucosyl transferase

The results of BLAST search of the cDNA clone of Red banana, amplified using UDP: glucose flavonoid 3-oxy-glucosyl transferase specific primer, showed significant similarity (Fig. 7) with *Vitis vinifera* mRNA for UDP: glucose 3-oxy-glucosyl transferase (Acc. No. AF000372). The cDNA synthesized from Green-red clone using UDP: glucose flavonoid 3 -oxy- glucosyl transferase specific primer exhibited similarity (Fig. 8) with *Vitis vinifera mRNA* (Table 6) for UDP: glucose 3-oxy-glucosyl transferase (Acc. No. AF000372).

The blastx search of cDNA clone from Red banana showed similarity with ceramide glucosyl transferase from *Synechocystis* sp. (BAA18121.1). cDNA clone from Green-red showed no significant similarity with any other reported proteins in the protein database (Table 7).

4. 1. 7 Comparison of the cDNA Sequences from Red and Green-red Clones

The sequence of cDNA clones obtained from Red and Green-red were compared using sequence alignment software ClustalX 1.81.

The cDNA clone from Red and Green-red amplified using chalcone synthase specific primer exhibited a maximum similarity when subjected to multiple sequence alignment followed by the cDNA clones amplified by UDP: glucose flavonoid 3-oxy-glucosyl transferase specific primer. The sequence alignment of cDNA clone amplified using dihydroflavonol 4- reductase specific primer showed least similarity.

The multiple sequence alignment result is presented in pages 40-41.

4.2 BIOCHEMICAL ANALYSIS

4.2.1 SDS-PAGE

Proteins isolated from Red banana and Green-red clone at three different stages including tissue culture shoots, leaves and fruit peel of field grown plants were separated on denaturing polyacrylamide gel to study the variation in protein profile. Gel electrophoresis of soluble proteins revealed no significant difference in total protein profile (Plate 9).

4.2.3 Estimation of Total Sugar

Total sugar was estimated using phenol sulfuric acid method. Total sugar content estimated in the peel, pulp, and peel together with the pulp of Red banana and Green-red clone showed slight difference (Table 9).

CLUSTAL X (1.81) result of multiple sequence alignment

A

```
Clone 1         TCTGATTTTCCTGTGCTGCGTG-TGTTTGTTGATCTTGCGTGTAGTGTTTGCAATCACGA
Clone 2         --CGATTTTCCTGTGCTGCGTGATGTTGTTTGTCCTGGTGTGTAGTGCGTGGAATC-TGT
                   ******************** ****  ***  ** * ********  ** ****  *

Clone 1         GTTTTGCTCCCTTCTCCCTTGCGAT-TGATAATGAGCCCTACCTCCACCACCATCCCCAA
Clone 2         CTTCGGACAGTTTCTCCCTTGCACTATGCCGGTGAGCCTTACCTCCACCCCCTTTCCTTA
                 **  *     ***********  * **    ****** ********** ** * **  *

Clone 1         GAACGACTAAAGTCTTACCCGCAAGGGGAGAAATAGATGCACCTTTTCCCGTGTGTTGGT
Clone 2         GTGCTACAAAAGTCTTACCCGCAAGGGGAGAAATAGATGCACCTTTTCCCGTGTGTTGCT
                *  * ** ************************************************** *

Clone 1         AGACC-
Clone 2         ACAATA
                * *
```

B

```
Clone 3         ----CATACGTTGTCGTGGAGGCGGGTTTCTCGGGGGCGTTTTCTTCCGGGCTCAGTCTA
Clone 4         TTTTCGTTCTTCCTCTTCCCGTGGGGTCTCTCGGGCTC-TGTCTGGACGGACTCGACTCA
                    * * * *  ** *   *  **** *******  * * *     *** ***     *

Clone 3         TTCCTGTAGTGGTTCCGGTGGGTTCACTCCTTTCCCCTCTACTCACGTCCGGGTTGTCGA
Clone 4         --CCAGTATTGTTTCGGTTCTGGAC-CCGCTATCTCTTGCTGTCTCATTCCCCTCTTCTC
                  ** *** ** *** * *  *  * *  ** ** * *    ** * * *   *  **

Clone 3         CTTTCGGAGGTGATGTTGTGCCTCTGATCAGCCCTCTTCTCCTAGAGTCTTGTATTCCGA
Clone 4         CTTATAGTTGT----TGGAAAGGCTGGCTA--CCTGTTCAGATCAGGATCCCCCTACCAC
                ***   *  **    * *     ***   *  *** ***   *   *       * **

Clone 3         TTTGTCCTATTCTTATTGTTGACGCGATGTATCCTTCCTTCTATTCTTATTCCTATCCTC
Clone 4         TTAATTCCCCCTTCATC-TCCACGTGAA----CCTCCCTGCTGCACTTCTTCTT--CTTC
                **  * *     * **  *  *** **     *** *** **   *** *** *  * **

Clone 3         TGTTATCCTGACCACTCTCCCCCCTCCCTTTTCGACTTTGCGATGTTCCTTTTCCCGGCC
Clone 4         CGGGGTCCT--TCTCAACTCCTCCTAAGGGGTGGGGGGGGGGGGGAAGAGGGAGGGGGGG
                 *   ****   * *    ** ***      * *     * *  *           **

Clone 3         TAAGTGGCCTAAAAGGTACTATGAATTCTTTTCCTCCCCCTTCATTCTTACTTACAGG
Clone 4         AAGGGGGGGAAAAATATATAGGGGAGGGGAGGGAGGTGCCGGTT--------------
                 * * **   ****  **    * *
```

C

```
Clone 5         GTTGCATCTTGTGCTGTGCCTTCTGGGGCTCTGTT-TCGTCCTGGCGATCTCTGCTGCCG
Clone 6         ------GCTTGTCTCCCCGCTTTTCTGTCTATTCGATCGTACTTGCCTTCCCTGCTGCCT
                      *****       *** *  * ** *    **** ** **  ** ********

Clone 5         TATCTAGGCGGCGAAGTAGTGCTCATCCTGACTTACTTCACTATATGTTATATATTTAAG
Clone 6         TATCT-GCCTGCTAGACCAT-CTCATCTTAACTTTCCG-GCTAGAACTTATATAGTAACA
                ***** * * ** *     * ****** * **** *    *** *  ******* * *
```

```
Clone 5         CATGCACTTCCCGACTTTTGTTCTTCAAATAATGACTATACGCCTGTAGCTACTGATCTA
Clone 6         TCTCTGCTTCCATATCTGTGTTCTTCAAATAACGACTATAGGCCTGTAGCTACTGATCTA
                  *   *****  *  * ************** ******* ******************

Clone 5         GCATCTTGCTAAGTGTATTCAACTGGTTAGCCTTCTATACTAGTTAGCCTCAAACTGACA
Clone 6         GCATCTTGCTAATTGTATTCAACTGGTTAGCCTTCTATACTAGTTAGTAGCAAACTGACA
                ************ ***********************************   **********

Clone 5         ATGCTTTATCCCATCTCTGCTGCCATATCTGTAAG-ATATGGAAGCGAAGATGAGTTGGA
Clone 6         ATGCTTTATCCCATCTCTGCTGCCATATCTGTAACTATGTGGTGACCTTATTGCTTTCTT
                ********************************** ** ***   *     **  **

Clone 5         TCCAAGACCACTACCACCTTCGAAGATGGAGGCGATAGGATGGGAAATCAGGCGGAGGAA
Clone 6         TCCAGTCTTAATGGAGAG---GAAAATAGGAGAAACTCATCGGTATACCAAGGAACT---
                ****     * *         *** ** *  *  *      ** * * ** *

Clone 5         GGC
Clone 6         ---
```

A cDNA clones amplified with chalcone synthase specific primer

B cDNA clones amplified with dihydroflavonol 4- reductase specific primer

C cDNA clones amplified with UDP: glucose 3- oxy- glucosyl transferase specific primer

Clone 1, 3, 5 represent Red banana
Clone 2, 4, 6 represent Green-red clones

Plate 9

Protein profile of different stages of Red banana and Green-red clone

Lane 1: Protein molecular weight marker

Lane 2: Tissue culture shoots of Red banana

Lane 3: Tissue culture shoots of Green-red clone

Lane 4: Leaf tissues of Red banana

Lane 5: Leaf tissues of Green-red clone

Lane 6: Fruit of Red banana

Lane 7: Fruit of Green-red clone

Lane 8: Protein molecular weight marker

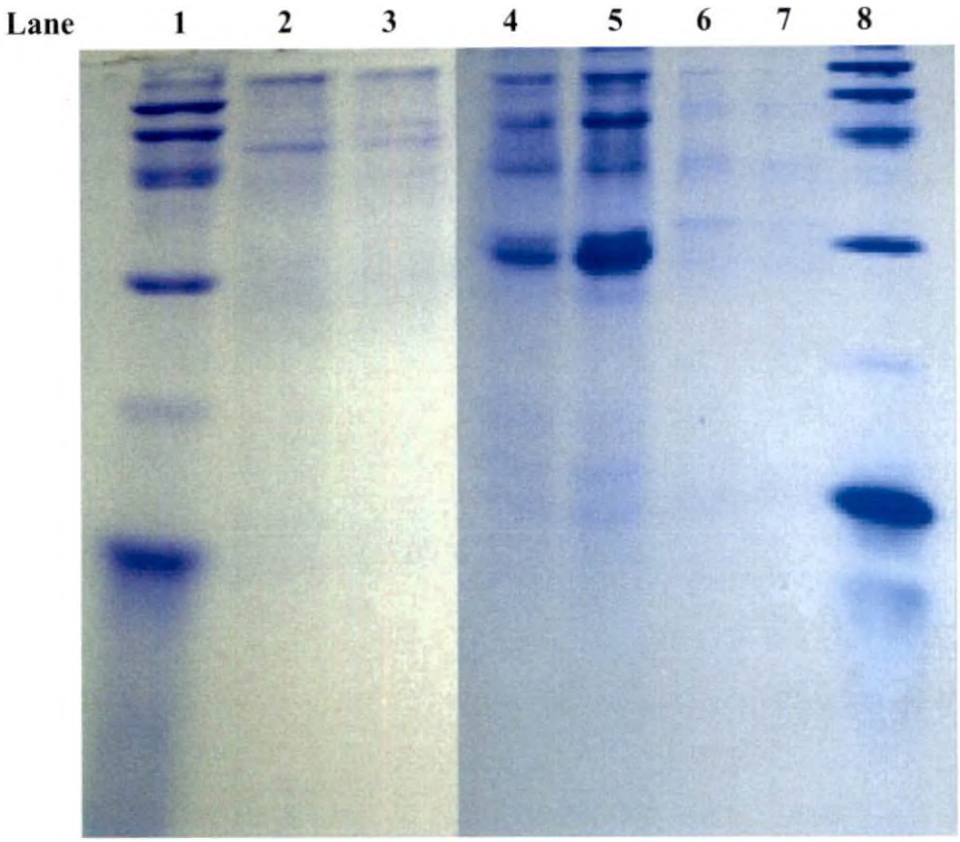


Plate 9. Protien profile of different stages of Red banana and Green-red clone

Table 2. Results of primer designing

Sl.No	Gene	Tm	GC%	Pair any	Pair 3'	Primer length	Sequence	Source plant
1	CHS	60.00 61.66	52.38 52.38	3.00	0.00	21 21	GACAAGTCGCAGATCAGGAAG CTACCAACACACGGGAAAAGG	*Oryza sativa*
2	CHS	60.34 60.33	52.38 50.00	4.00	0.00	21 20	ATCACGCACCTAGTGTTCTGC CCATGCGGTTTTATGACTCC	*Zea mays*
3	CHS	60.50 60.40	52.38 52.38	5.00	1.00	21 21	AGAGGAGAACCCCAACATGAG CCACTGCACTGTGTTGACTTG	*Sorghum bicolor*
4	CHS	60.14 60.60	52.38 52.38	4.00	0.00	21 21	CACCACCCAACTGTATTGACC CCAAATAGAACACCCCACTCC	*Fragaria ananasa*
5	DFR	60.71 61.76	55.56 54.55	3.00	0.00	18 22	AATAAAGGGCCGGTGGTG GAGCTTCTTGGAGGAGAAGTGG	*Triticum aestivum*
6	DFR	60.26 59.27	52.63 52.38	4.00	0.00	19 21	GCGAATCCAACACAAGCAC AGCGTGTACCTGAACCTGAAC	*Oryza sativa*
7	DFR	59.84 58.60	57.14 50.00	4.00	1.00	21 20	GGGTTATGAGGTCAGGGCTAC CAGCTGCTGAGACCATTTTC	*Oncidium* clone
8	DFR	59.86 60.29	57.14 52.38	6.00	2.00	21 21	AGACTGAGAGGAAGGGTCCAG GAGAAGCTCACGCTCTCAATG	*Cymbidium* hybrid
9	UFGT	60.77 60.33	52.38 52.38	4.00	1.00	21 21	CAGATATGGCAGCAGAGATGG GGCTCTCAGATTTTCCCTCAG	*Vitis vinifera*
10	UFGT	60.23 59.99	55.00 50.00	3.00	1.00	20 20	GTATCCCTGGTTTGGTGTCG TCCATCACAGCTTTCACTGC	*Ipomoea purpurea*
11	UFGT	60.51 60.37	52.38 52.38	6.00	0.00	21 21	CGCCAAGAGACTCCTAAAAGC CTTCTCTGGCCAAAGTCTTCC	*Perilla frutescens*
12	UFGT	60.02 59.79	52.38 52.38	3.00	1.00	21 21	CCAACAGCTCAATCCTAGCAG TCCACACATCCTCTACCATCC	*Fragaria ananasa*

Table 3. Clone sequence data

Sl. No.	Name of gene/ clone	Sequence length (bp)	Sequence (5'→3')
1.	CHS Red banana	183	TCTGATTTTCCTGTGCTGCGTGTGTTTGTTGATCTTGCGTGTAGTGTTTGCAATCACGAGTT TTGCTCCCTTCTCCCTTGCGATTGATAATGAGCCCTACCTCCACCACCATCCCCAAGAACG ACTAAAGTCTTACCCGCAAGGGGAGAAATAGATGCACCTTTTCCCGTGTGTTGGTAGACC
2.	CHS Green-red	183	CGATTTTCCTGTGCTGCGTGATGTTGTTTGTCCTGGTGTGTAGTGCGTGGAATCTGTCTTC GGACAGTTTCTCCCTTGCACTATGCCGGTGAGCCTTACCTCCACCCCCTTTCCTTAGTGCT ACAAAAGTCTTACCCGCAAGGGGAGAAATAGATGCACCTTTTCCCGTGTGTTGCTACAATA
3.	DFR Red banana	354	CATACGTTGTCGTGGAGGCGGGTTTCTCGGGGGCGTTTTCTTCCGGGCTCAGTCTATTCCT GTAGTGGTTCCGGTGGGTTCACTCCTTTCCCCTCTACTCACGTCCGGGTTGTCGACTTTCG GAGGTGATGTTGTGCCTCTGATCAGCCCTCTTCTCCTAGAGTCTTGTATTCCGATTTGTCC TATTCTTATTGTTGACGCGATGTATCCTTCCTTCTATTCTTATTCCTATCCTCTGTTATCCT GACCACTCTCCCCCCTCCCTTTTCGACTTTGCGATGTTCCTTTTCCCGGCCTAAGTGGCCT AAAAGGTACTATGAATTCTTTTCCTCCCCCTTCATTCTTACTTACAGG
4.	DFR Green-red	325	TTTTCGTTCTTCCTCTTCCCGTGGGGTCTCTCGGGCTCTGTCTGGACGGACTCGACTCACC AGTATTGTTTCGGTTCTGGACCCGCTATCTCTTGCTGTCTCATTCCCCTCTTCTCCTTATAG TTGTTGGAAAGGCTGGCTACCTGTTCAGATCAGGATCCCCCTACCACTTAATTCCCCCTTC ATCTCCACGTGAACCTCCCTGCTGCACTTCTTCTTCTTCCGGGGTCCTTCTCAACTCCTCCT AAGGGGTGGGGGGGGGGGGGAAGAGGGAGGGGGGGAAGGGGGGGAAAAATATATAGGG GAGGGGAGGGAGGTGCCGGTT
5.	UFGT Red banana	361	GTTGCATCTTGTGCTGTGCCTTCTGGGGCTCTGTTTCGTCCTGGCGATCTCTGCTGCCGTATC TAGGCGGCGAAGTAGTGCTCATCCTGACTTACTTCACTATATGTTATATATTTAAGCATGCA CTTCCCGACTTTTGTTCTTCAAATAATGACTATACGCCTGTAGCTACTGATCTAGCATCTTGC TAAGTGTATTCAACTGGTTAGCCTTCTATACTAGTTAGCCTCAAACTGACAATGCTTTATCCC ATCTCTGCTGCCATATCTGTAAGATATGGAAGCGAAGATGAGTTGGATCCAAGACCACTACC ACCTTCGAAGATGGAGGCGATAGGATGGGAAATCAGGCGGAGGAAGGC
6.	UFGT Green-red	345	GCTTGTCTCCCCGCTTTTCTGTCTATTCGATCGTACTTGCCTTCCCTGCTGCCTTATCTGCCTGC TAGACCATCTCATCTTAACTTTCCGGCTAGAACTTATATAGTAACATCTCTGCTTCCATATCTG TGTTCTTCAAATAACGACTATAGGCCTGTAGCTACTGATCTAGCATCTTGCTAATTGTATTCA ACTGGTTAGCCTTCTATACTAGTTAGTAGCAAACTGACAATGCTTTATCCCATCTCTGCTGCC ATATCTGTAACTATGTGGTGACCTTATTGCTTTCTTTCCAGTCTTAATGGAGAGGAAAATAGGAG AAACTCATCGGTATACCAAGGAACT

Table 4. Result for blastn similarity search for the clones amplified with chalcone synthase specific primer

Sl.No	Clone	Target gene	Clone origin	Similar accessions.	Accession No.
1.	Clone 1	Chalcone synthase	Red banana	*O. sativa* mRNA for chalcone synthase	X89859
				N. tabacum mRNA for cysteine-rich extensin-like protein	L13440
				Oryza sativa (japonica cultivar-group), mRNA	XM_470819.1
				Arabidopsis thaliana genomic DNA, chromosome 5, P1 clone	AB016891.1
2.	Clone 2	Chalcone synthase	Green-red	*Medicago truncatula* clone mth2-23o13, complete sequence	AC14459.8
				Arabidopsis thaliana unknown protein AT4G09150 mRNA, complete cds	NM116984.3
				Arabidopsis thaliana DNA chromosome 4, contig fragment No. 26	AL161514.2
				Arabidopsis thaliana Full-length cDNA Complete sequence from clone GSLTSIL79ZB08 of Silique of strain col-0 of *Arabidopsis thaliana* (thale cress)	BX829182.1

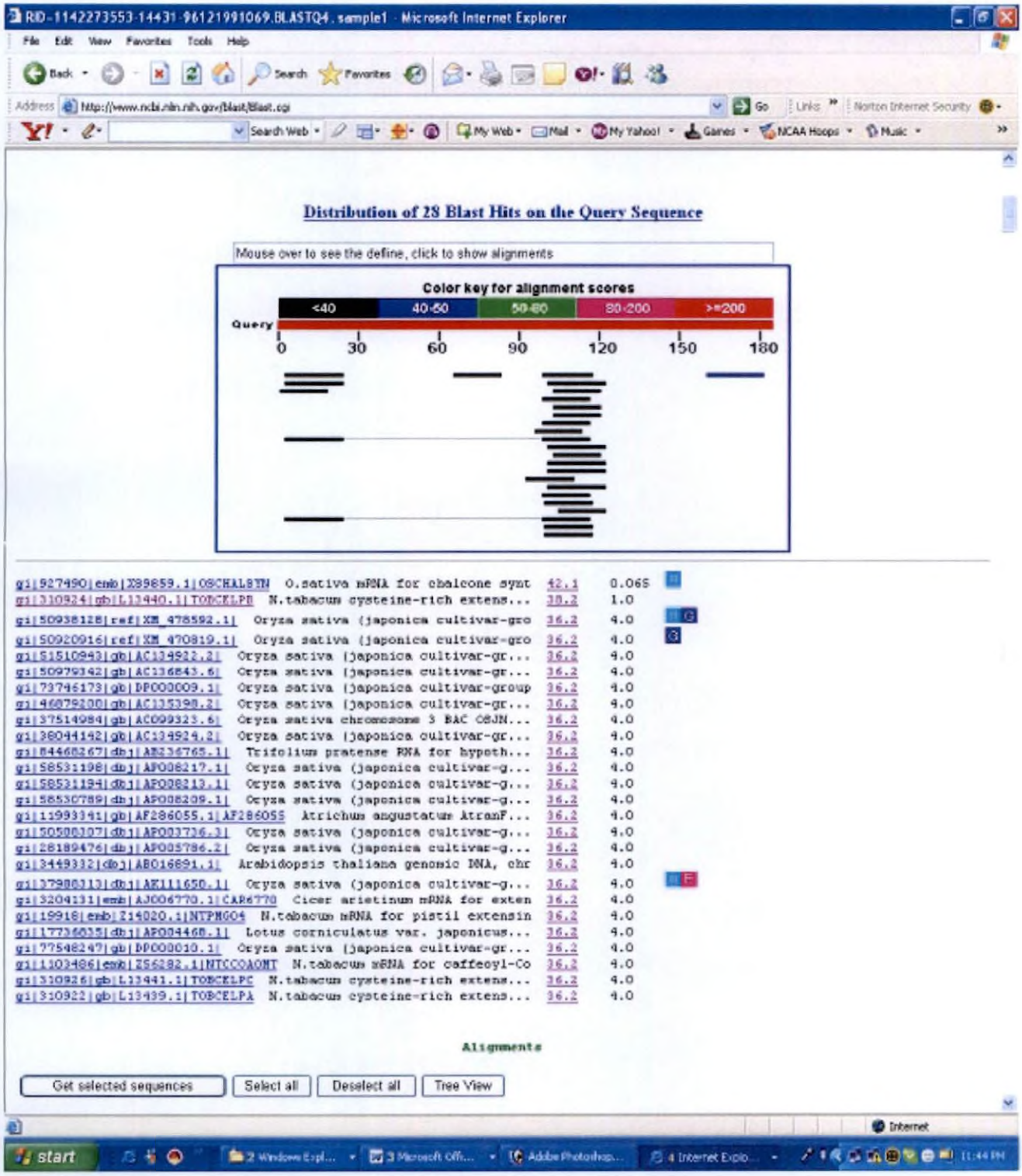


Fig. 3. Blastn result for cDNA clone from Red banana amplified with chalcone synthase specific primer

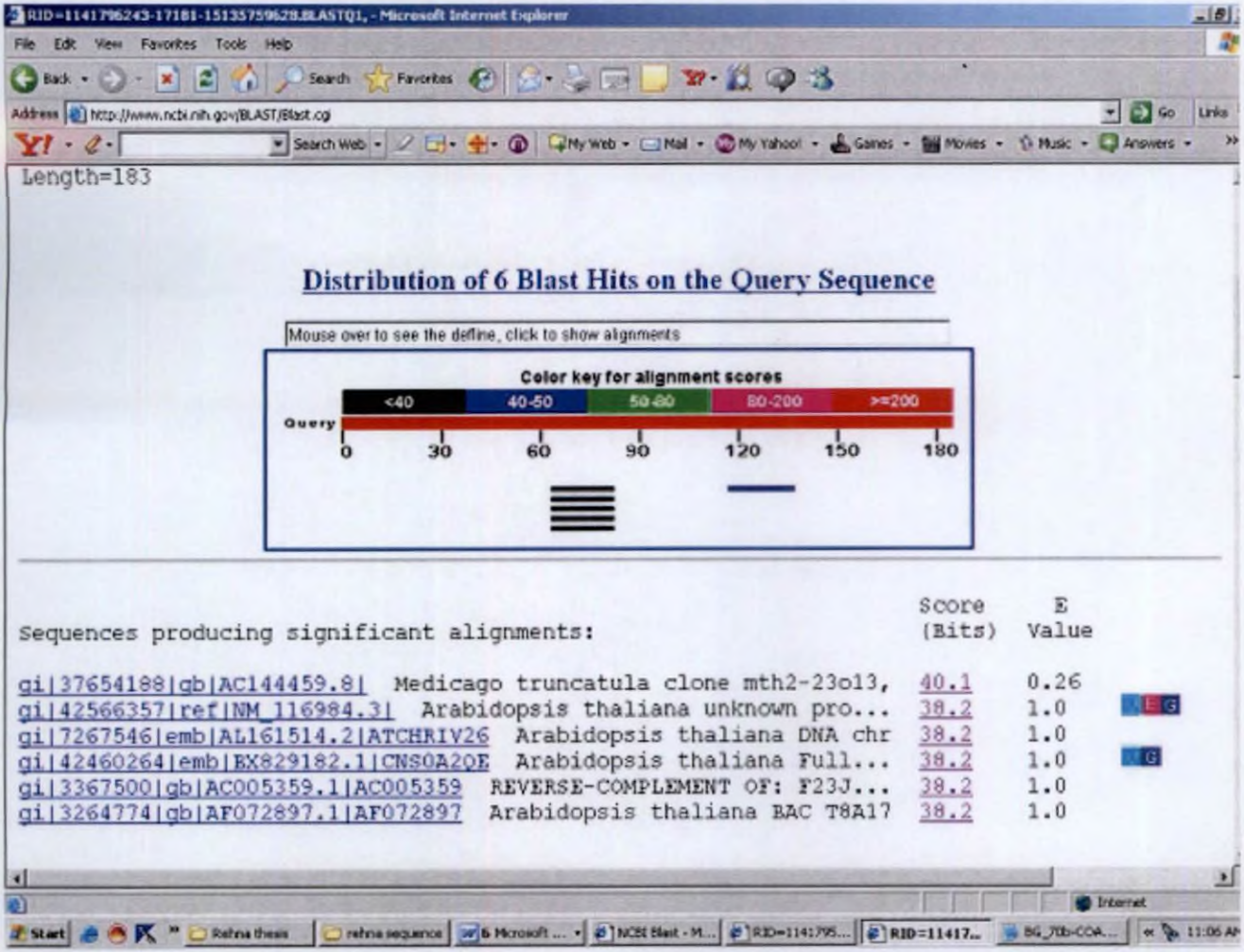


Fig. 4. Blastn result for cDNA clone from Green banana amplified with chalcone synthase specific primer

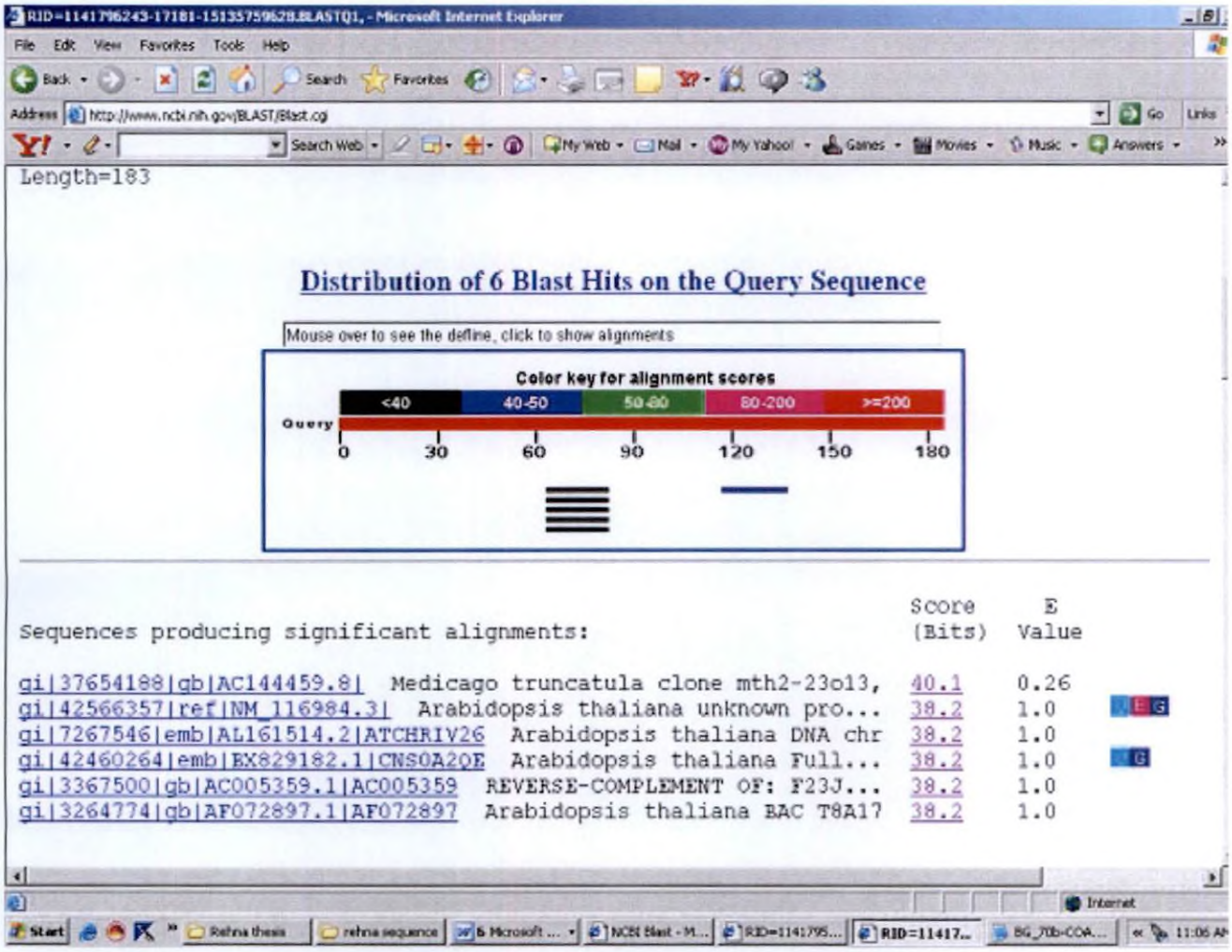


Fig. 4. Blastn result for cDNA clone from Green banana amplified with chalcone synthase specific primer

Table 5. Result for blastn similarity search for the clones amplified with dihydroflavonol 4 - reductase specific primer

Sl. No	Clone	Target gene	Clone origin	Similar accessions.	Accession No.
1.	Clone 3	Dihydroflavonol 4 - reductase	Red banana	*Triticum aestivum* plasma membrane H+-ATPase gene, *Medicago truncatula* clone mth2-17c2, *Arabidopsis thaliana* unknown protein AT3G56590 mRNA, *Oryza sativa* (japonica cultivar-group) chromosome 11 clone OSJNBa0008E02,	AY829002 AC144766.18 NM 115518.2 AC135497.3
2.	Clone 4	Dihydroflavonol 4 - reductase	Green-red	*Physcomitrella patens* cytokinin receptor 1 (CRE1) gene *Oryza sativa* (japonica cultivar-group), mRNA *Arabidopsis thaliana* unknown protein AT1G10890 mRNA, *Oryza sativa* (japonica cultivar-group) chromosome 8 BAC OSJNBa0023H09	DQ389162.1 XM468560.1 NM100964.1 AY360391.1

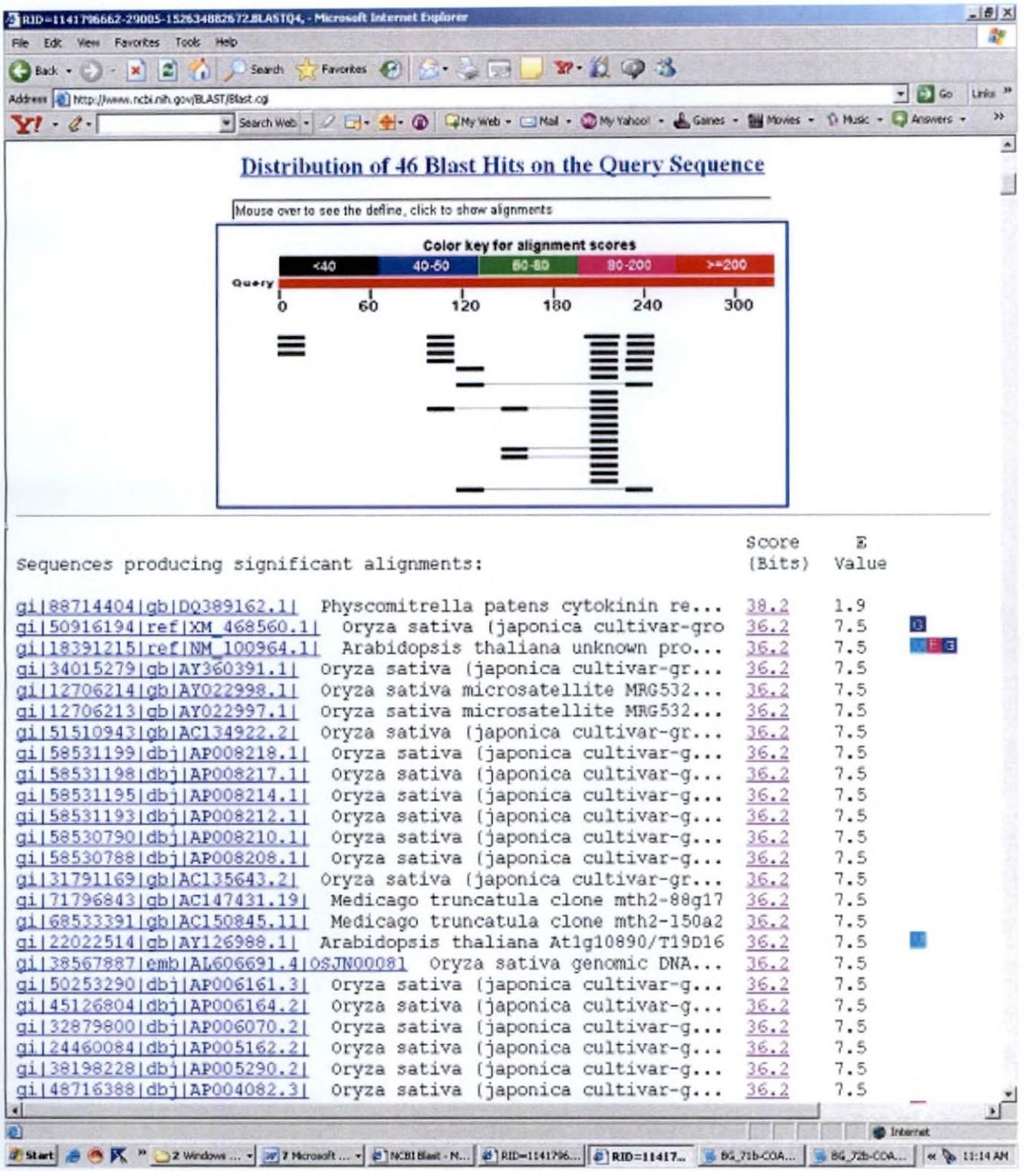


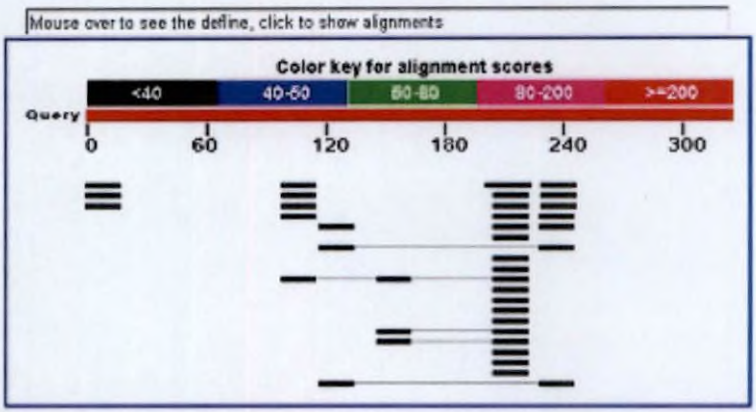
Fig. 5. Blastn result for cDNA clone from Red banana amplified with dihydroflavonol 4-reductase specific primer

RID=1141796662-29005-152634882572.BLASTQ4, - Microsoft Internet Explorer

File Edit View Favorites Tools Help

Address http://www.ncbi.nih.gov/BLAST/Blast.cgi

Distribution of 46 Blast Hits on the Query Sequence



Sequences producing significant alignments:		Score (Bits)	E Value
gi|88714404|gb|DQ389162.1|	Physcomitrella patens cytokinin re...	38.2	1.9
gi|50916194|ref|XM_468560.1|	Oryza sativa (japonica cultivar-gro	36.2	7.5
gi|18391215|ref|NM_100964.1|	Arabidopsis thaliana unknown pro...	36.2	7.5
gi|34015279|gb|AY360391.1|	Oryza sativa (japonica cultivar-gr...	36.2	7.5
gi|12706214|gb|AY022998.1|	Oryza sativa microsatellite MRG532...	36.2	7.5
gi|12706213|gb|AY022997.1|	Oryza sativa microsatellite MRG532...	36.2	7.5
gi|51510943|gb|AC134922.2|	Oryza sativa (japonica cultivar-gr...	36.2	7.5

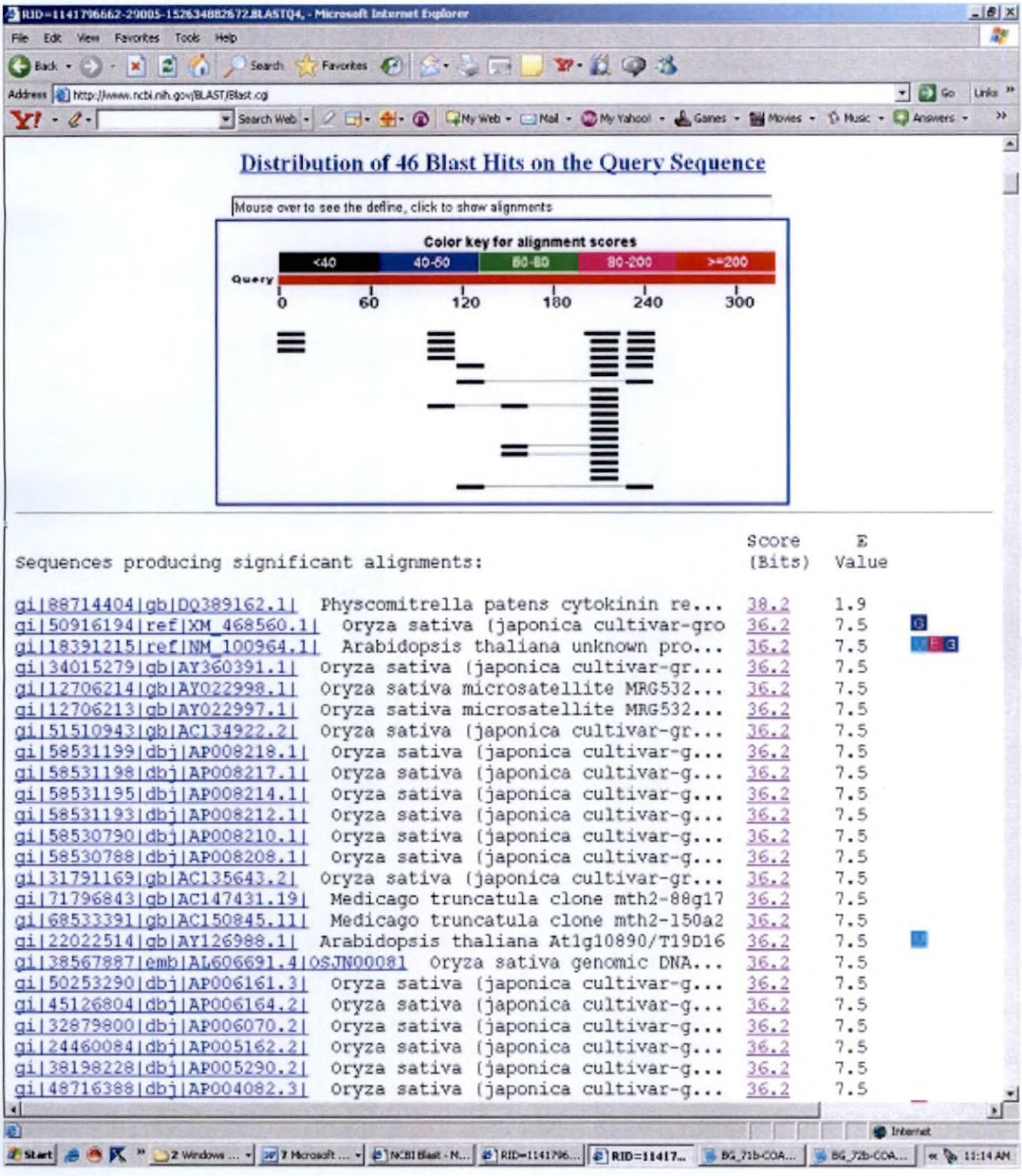


Fig. 6. Blastn result for cDNA clone from Green-red amplified with dihydroflavonol 4-reductase specific primer

Table 6. Result for blastn similarity search for the clones amplified with UDP: glucose 3-oxy- glucosyl transferase specific primer

Sl. No	Clone	Target gene	Clone origin	Similar accessions.	Accession No.
1.	Clone 5	UDP: glucose 3-o-glucosyl transferase	Red banana	*Vitis vinifera* mRNA for UDP: glucose 3-o-glucosyl transferase *Vitis vinifera* mRNA for UDP: glucose 3-o-glucosyl transferase *Vitis vinifera* mRNA for UDP: glucose 3-o-glucosyl transferase *Vitis vinifera* mRNA for UDP: glucose 3-o-glucosyl transferase	AF000372 AF000371 AB047099.1 AB047098.2
2.	Clone 6	UDP: glucose 3-o-glucosyl transferase	Green-red	*Vitis vinifera* mRNA for UDP: glucose 3-o-glucosyl transferase *Vitis vinifera* mRNA for UDP: glucose 3-o-glucosyl transferase *Vitis vinifera* mRNA for UDP: glucose 3-o-glucosyl transferase *Vitis vinifera* mRNA for UDP: glucose 3-o-glucosyl transferase	AF000372 AF000371 AB047099.1 AB047098.2

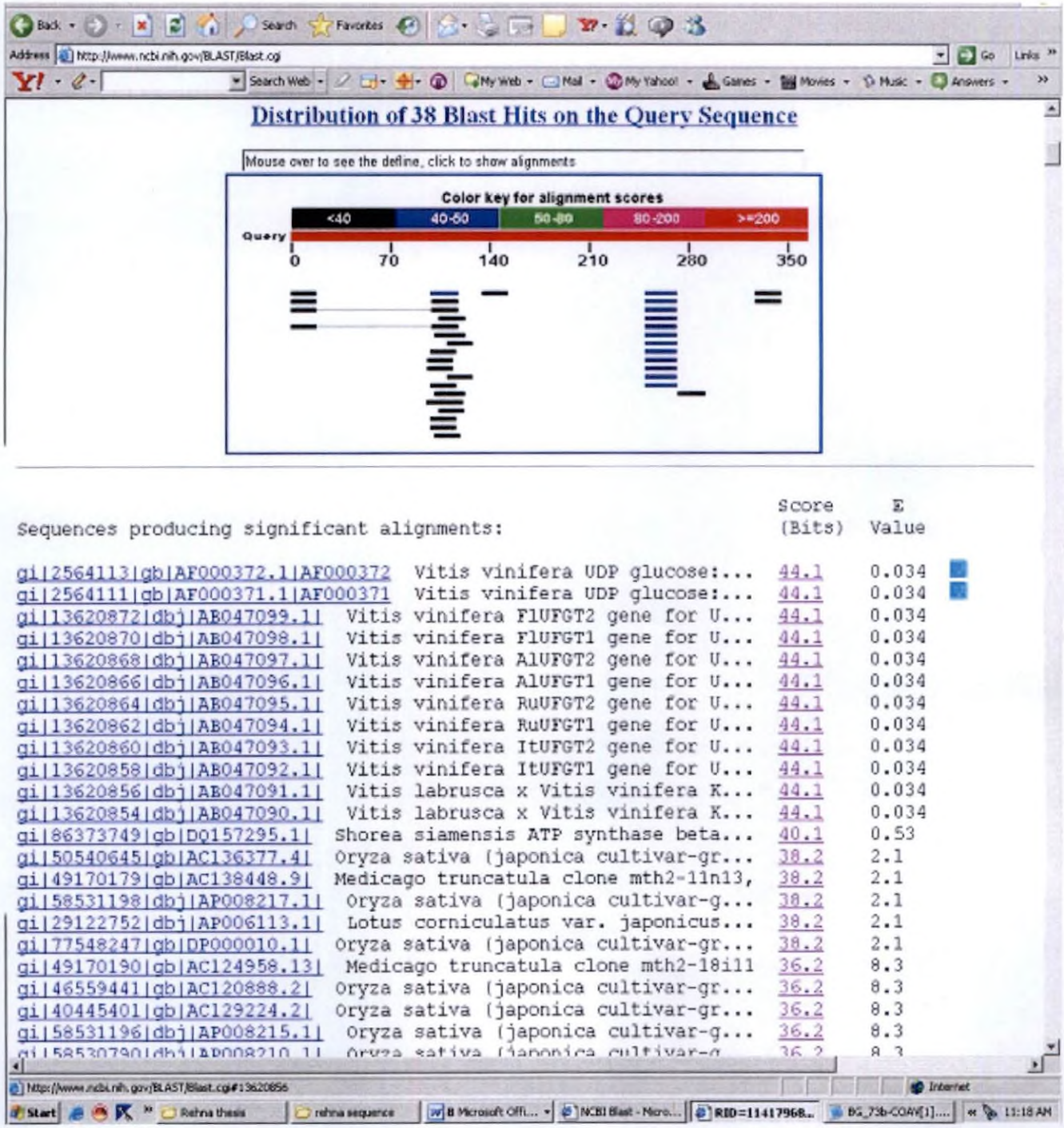


Fig. 7. Blastn result for cDNA clone from Red banana amplified with UDP: glucose 3-oxy-glucosyl transferase specific primer

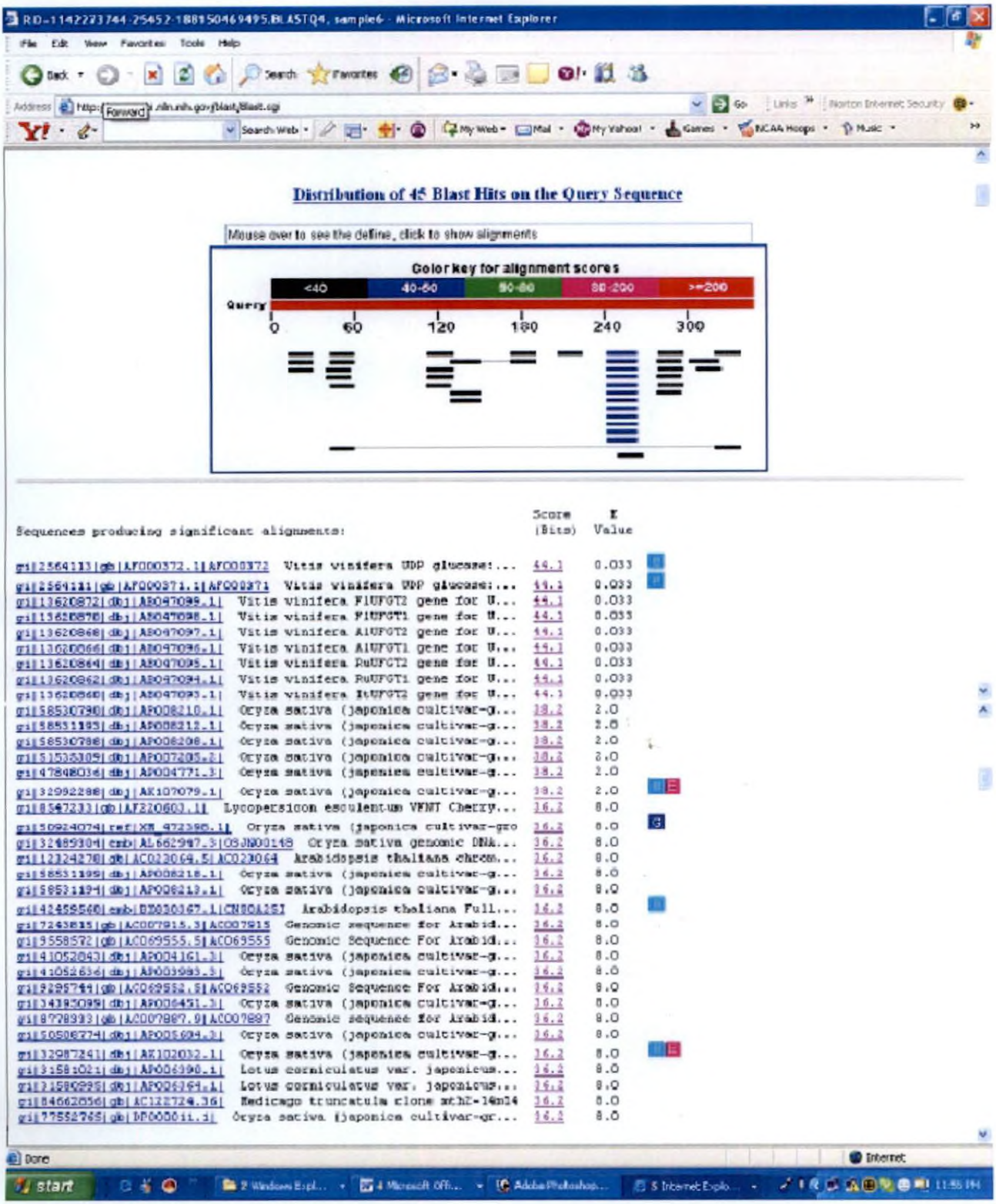


Fig. 8. Blastn result for cDNA clone from Green-red amplified with UDP: glucose 3-oxy-glucosyl transferase specific primer

Table 7. Result for blastx similarity search for the clones

Clone No.	Clone origin	Expected enzyme	Observed similarity	Accession No.	Source organism
1.	Red banana	Chalcone synthase	Shikimate 5 – dehydrogenase	NP_918761	*O. sativa*
2.	Green-red		Putative 3-dehydroquinate dehydratase/ shikimate 5-dehydrogenase	NP_918759	*O. sativa*
3.	Red banana	Dihydroflavanol 4 - reductase	Hypothetical protein, 5'-partial	AAL79707	*O. sativa*
4.	Green-red		OSJNBa0053B21.12	CAE05538	*O. sativa*
5.	Red banana	UDP: glucosyl 3 –o-transferase	Ceramide glucosyltransferase	BAA18121.1	*Synechocystis* sp.
6.	Green-red		No significant similarity found		

Table 8. Total sugar content of Red banana and Green-red clones

Sl. No	Clones	Peel (%)	Pulp (%)	Peel + Pulp (%)
1.	Red banana	13.48	13.19	13.15
2.	Green-red	13.29	12.78	13.09

Discussion

5. DISCUSSION

Red banana, one of the important commercial banana varieties, is grown in southern Kerala and adjoining areas of Tamil Nadu. The fruits are highly priced, compared to that of other varieties. Red banana belongs to AAA genome. The plants are robust and generally free of pests and diseases, especially viral diseases. A serious problem faced by the growers of this variety is the irreversible colour change of Red banana to green in a sizeable number of population. The green variant, Green-red fetches low price in the market, as the consumer preference is for the red colour. The first mention about the colour change was made by Simmonds (1959). According to him, the colour change is a developmental accident rather than mutation. Not much study has been made on this aspect to understand the cause.

Factors affecting fruit colour are primarily genetically determined. In addition, environmental factors such as nutrients, temperature and light conditions can have an effect on flavonoid composition and final colour of the fruit (Jaakola, 2003). The chance for involvement of environmental factors can be ruled out in the case of Red banana because the change can be observed in plants grown in the same field under same environmental and soil conditions. The change occurs even in tissue culture flasks in which all the conditions are controlled.

The colour of Red banana 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. Also the role of gene promoters has been suggested by Quattrachio *et al.* (1998). They indicated that the regulatory genes of anthocyanin pathway are conserved across species and that divergent evolution of flavonoid biosynthesis gene promoters might be responsible for the species specific difference in regulatory networks.

In this context, a study was undertaken with the objective of differentiating the Red banana and Green-red clone based on the expression of genes involved in anthocyanin pigmentation. Three key enzymes *viz.,* chalcone synthase (CHS), dihydroflavonol 4- reductase (DFR), and UDP: glucose flavonoid 3-oxy-glucosyl transferase (UFGT) of anthocyanin biosynthetic pathway were selected. The research also focused on isolation and characterization of these three genes from the two clones using RT-PCR. Differentiation of the two clones based on total protein profile and sugar content was also aimed at.

For isolating RNA, seven protocols were tried. Some of them were completely unsuccessful, whereas the others gave low yields of more or less impure RNA. The most common RNA isolation method using guanidium thiocyanate failed to recover RNA after ethanol precipitation. RNA isolated with CTAB buffer, followed by phenol-chloroform extraction, was highly contaminated with genomic DNA. Method developed by Venkatachalam *et al.* (1999) worked well with leaf tissues but was unsuccessful with fruit tissues. It may be due to the presence of large quantities polysaccharides and polyphenolic compounds. Similar problem was also reported by Liu *et al.* (1998). The method developed by Schneitz (2000) was found to be successful for isolation of good quality RNA from both fruit peel and leaf tissues of the two clones in the present study. The method utilized hot phenol maintained at 80°C for reducing polysaccharide interference. Ainsworth (1994) also used high incubation temperature for isolating good quality RNA from banana fruit tissues. Addition of PVP, 1.5 per cent in the extraction buffer could overcome the problem of polyphenol interference. The problem of residues of coloured substances which binds to RNA to form insoluble complexes (Pawlowski *et al.,* 1994) could be overcome by additional washing steps with ethanol after the first precipitation of RNA with lithium chloride. RNA yields from fruit tissues were constantly lower, compared to other tissues.

Agarose gel electrophoresis of RNA 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.

The polyphenols and polysaccharides residues in the fruit tissues could not be completely removed during mRNA purification. However, the residues did not interfered with enzyme activity during reverse transcription. Heterologous primers were designed for the specific amplification of chalcone synthase, dihydroflavonol 4-reductase and *UDP*: glucose 3-oxy-glucosyl transferase since no reports were available on the isolation of anthocyanin pigmentation genes from banana. Primer designing software primer3 was used for this purpose. Two primer pairs were selected for each gene.

Reverse transcription-PCR was carried out for cDNA amplification. During the reaction non specific amplification was observed initially. This problem was solved by decreasing primer concentration and increasing annealing temperature.

The cDNA amplified from both Red banana and Green-red clones with chalcone synthase specific primers showed a single band of approximately 180 bp which were cloned and sequenced for further analysis. The result showed that the cDNA fragment amplified was of 183 bp length. Homology search of cDNA clone from Red banana showed similarity with mRNA for chalcone synthase isolated from rice. For Green-red cDNA clone, no similarity was observed with other chalcone synthase sequences in the Gen Bank. Multiple sequence alignment of the clones representing chalcone synthase showed slight differences in the sequence between the two. However, the translated query *vs.* protein database search (blastx) of both clones exhibited similarity with shikimate dehydrogenase (SDH), an enzyme involved in the biosynthesis of aromatic amino acids. Even though the nucleotide sequences were dissimilar protein coded by the two was the same. This may be attributed to the degenerate nature of codons.

Reverse transcription polymerase chain reaction of Red banana and Green-red clone using dihydroflavonol 4- reductase specific primer yielded a reproducible fragment of size approximately 300 bp which was eluted, cloned and sequenced. The result showed a fragment of length of 354bp and 325bp in Red banana and Green-red clone respectively. Similarity search of cDNA from both Red banana and Green-red clone showed no significant similarity with any of the dihydroflavonol 4- reductase sequences in the Gen Bank. The translated query *vs.* protein database also showed no similarity with any of the dihydroflavonol 4- reductase protein sequences. The sequence of cDNA showed mononucleotide repeats. This may be because the primer designed was not specific. The heterologous primers may not be always specific.

The cDNA product of approximately 350 bp, amplified from Red banana and Green- red clones, with UDP: glucose 3-oxy-glucosyl transferase specific primer, was cloned and subjected to BLAST analysis. The blastn search showed similarity with UDP: glucose 3-oxy-glucosyl transferase from *Vitis vinifera* for both Red and Green-red clone. The translated query *vs.* protein database search of Red banana clone showed similarity with ceramide glucosyl transferase from *Synechocystis* sp. whereas Green-red clone showed no similarity with any of the glucosyl transferase. This indicates certain modifications to UDP: glucose 3-oxy-glucosyl transferase of Green-red clone during translational or post translational events. To confirm this assumption further investigations are needed.

The problem of colour change is observed in a wide variety of other plants such as strawberry, bilberry, grapes, *Perilla frutescence*, *Forsythia intermedia*, petunia, tomato, sweet orange, *Antirrhinum majus* etc. Anthocyanin biosynthesis has been extensively studied in these plant species, and therefore, detailed information on the course of reaction is available. On the other hand, in Red banana the anthocyanin pigmentation pathway is not yet studied. Enormous research has been conducted on environmental and developmental regulation of a wide range of genes, encoding

enzymes in metabolic pathways. At least 30 genes are believed to be involved in flower pigmentation (Kroon *et al.*, 1994). The regulatory mechanism of the pathway varies from plant to plant. For instance, in *Petunia,* regulation occurs from DFR onwards. In *Antirrhinum* regulation occurs one step ahead *viz.,* from flavonoid 3-hydroxylase (F3H) onwards. In Red banana, understanding anthocyanin biosynthetic pathway and regulation of genes involved is necessary to draw meaningful conclusions.

One of the major factors that determine pigmentation is sugar concentration (Courteny-Gutterson, 1994). Partitioning of photosynthates between peel and pulp of the fruit can also affect pigmentation. Hence, sugar content was estimated in both pulp and peel of the two clones. Though there was slight variation in the total sugar content between the peel and pulp tissues, the difference was not significant. This suggested that there was no significant difference in the partitioning of the photosynthate between peel and pulp. Increased sugar concentration increases pigmentation but it fails to colour the mutants. Total protein profile obtained by PAGE of Red banana and Green-red clone were also similar.

Altered regulation of various structural and regulatory genes involved in the biosynthesis and accumulation of anthocyanin could be the reason for the formation of Green-red clone. The data generated by this study is not sufficient enough for arriving at a conclusion. The RT-PCR studies revealed that the genes selected for the study were expressed in both Red and Green-red. For the comparison of nucleotide sequences and translated sequences, full length cDNA is necessary. But RT-PCR provides only partial cDNA. For the better understanding of this phenomenon of colour change, detailed studies similar to those conducted in other plants are needed to be conducted in Red banana too. Instead of heterologous primers degenerate primers can be used for gene amplification. Methods like RACE and Real time PCR may be helpful in analyzing the difference in the expression of genes. Enzyme assays can be done for understanding the activity of enzymes in a better way.

6. SUMMARY

The study entitled "Differential expression of genes involved in anthocyanin pigmentation in Red banana and Green-red clones" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2003 to 2006. The objective of the study was to analyze the expression of three key enzymes *viz.*, chalcone synthase (CHS), dihydroflavonol 4-reductase (DFR), and UDP: glucose 3-oxy-glucosyl transferase (UFGT) in Red banana and Green-red clones. The study was also aimed at differentiating the two clones based on biochemical characters such as total protein profile and sugar content. The clones at three different stages, tissue culture shoot, leaf as well as fruit of field grown plants, were subjected to analysis. The salient findings of the study are summarized below.

Extraction of RNA from fruit tissues was extremely laborious due to the presence of secondary metabolites and polysaccharides. Extraction protocol utilizing heated phenol followed by lithium chloride precipitation was the best method for isolation of RNA from banana fruit. Additional washing with 70 per cent ethanol could improve the quality of RNA.

Since, there was no report on the isolation of anthocyanin genes in banana, heterologous primers were designed based on the gene sequence from strawberry and rice. RT-PCR using primers designed with rice chalcone synthase mRNA sequences gave better amplification of cDNA (approximately 180 bp) isolated from Red banana and Green-red clones, compared to primers based on strawberry gene sequences. The primers designed for dihydroflavonol 4-reductase based on rice mRNA sequence for dihydroflavonol 4-reductase gave good amplification of cDNA (approximately 300 bp) isolated from Red banana and Green-red clones. UDP: glucose 3-oxy-glucosyl transferase amplified using primer designed from grapes mRNA sequence gave better amplification of cDNA (approximately 350 bp) in both Red and Green-red clone.

The sequence of chalcone synthase cDNA clone of Red banana showed similarity (blastn) with *Oryza sativa* (Acc. No.X89859) mRNA for chalcone synthase. Blastn search of cDNA clone from Green-red showed similarity with *Medicago truncatula* clone mth2-23o13 (Acc. No. AC14459.8). Translated query *vs.* protein database (blastx) search of both Red and Green-red showed similarity with the enzyme shikmate 5 –dehydrogenase/ 3-dehydroquinate dehydratase from *Oryza sativa* (japonica cultivar-group).

The blastn search of the cDNA clone of Red banana and Green-red clones, amplified using dihydroflavonol 4- reductase specific primer, showed no significant similarity with other dihydroflavonol 4- reductases available in the Gen Bank. The translated query *vs.* protein database search revealed similarity with a hypothetical protein, 5'-partial from *Oryza sativa* ((japonica cultivar-group, Acc. No. AAL79707) for cDNA clone of Red banana. The translated query *vs.* protein database search of cDNA from Green-red clone revealed similarity with OSJNBa0053B21.12 from *Oryza sativa* ((japonica cultivar-group, Acc. No. CAE05538).

The cDNA clone of both Red banana and Green-red, amplified using UDP: glucose flavonoid 3 -Oxy- glucosyl transferase specific primer, showed significant similarity with *Vitis vinifera* mRNA for UDP: glucose 3-oxy-glucosyl transferase (Acc. No. AF000372) during blastn. The blastx search of cDNA clone from Red banana showed similarity with ceramide glucosyl transferase from *Synechocystis* sp. (BAA18121.1). cDNA clone from Green-red showed no significant similarity with any other proteins in the database.

Sequence alignment using the software Clustal X 1.81 of the two clones showed slight dissimilarity. Highest similarity was observed with clones amplified using chalcone synthase specific primer followed by clones amplified with UDP: glucose 3-oxy-glucosyl transferase specific primer. Least similarity was observed for clones amplified using dihydroflavonol 4- reductase specific primers.

Polyacrylamide gel electrophoresis revealed no difference in the total protein profile of Red banana and Green-red clone at any stage of development. Total sugar content in the peel, pulp and peel together with the pulp of Red banana and Green-red clone showed slight variation which was not significant.

Gene expression analysis of Red banana and Green-red clones revealed no significant difference with respect to the expression of the three genes under study. The clones did not differ in total protein profile and total sugar content.

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Appendices

APPENDIX I

RNA Extraction buffer (pH 8.0)

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

APPENDIX II

S. O. C. medium

Tryptone	2.0%
Yeast extract	0.5%
NaCl	10mM
KCl	25mM
$MgCl_2$	10mM
$MgSO_4$	10mM
Glucose (Dextrose)	20mM
pH	7.0

Store at RT or +4°C

APPENDIX III

Plasmid isolation

1. Solution I

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

2. Solution II

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

3. Solution III

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

4. L. B medium (pH 7.0)

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

APPENDIX IV

Protein extraction buffer

Phosphate buffer (pH 7.0)	0.1M
EDTA	1.0M
PMSF	1.0M
BME	5.0mM
PVP	0.1%

APPENDIX V

Polyacrylamide gel electrophoresis

1. Resolving Gel (12%)

30% Acrylamide: bis- acrylamide (30: 0.8)	12.0ml
Tris – HCl (8.0)1.5M	7.5ml
10% SDS	0.3ml
10% Ammonium per sulphate	0.3ml
TEMED	0.012ml
H_2O	9.9ml
Total volume	30ml

2. Stacking Gel (5%)

30% Acrylamide: bis- acrylamide (30: 0.8)	1.0ml
Tris – HCl (6.8)1.0M	0.75ml
10% SDS	0.06ml
10% Ammonium per sulphate	0.06ml
TEMED	0.006ml
H_2O	4.1ml
Total volume	6ml

3. Stacking gel buffer

Tris – HCl (pH 6.8)	1.0M

4. Resolving gel buffer

Tris – HCl (8.0)	1.5M

5. Reservoir buffer
   Tris glycine (pH 8.3)

Tris base	3.0g
Glycine	14.4g
SDS	1.0g
Distilled water	1000ml

6. Sample buffer

Tris – HCl (pH 8.0)	0.0625M
SDS	5.0%
β – Mercaptoethanol	2.0%

Sucrose	10.0%
Bromophenol blue	0.002%

7. CBB staining solution

CBB R – 250	2.0g
Methanol	50.0ml
Glacial Acetic acid	10.0ml
H_2O	40.0ml
Total volume	100ml

8. Destaining solution

Methanol	50.0ml
Glacial Acetic acid	10.0ml
H_2O	40ml
Total volume	100ml

Abstract

DIFFERENTIAL EXPRESSION OF GENES INVOLVED IN ANTHOCYANIN PIGMENTATION IN RED BANANA AND GREEN-RED CLONES

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Abstract of the
thesis submitted in partial fulfilment of the requirement
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ABSTRACT

The study entitled "Differential expression of genes involved in anthocyanin pigmentation in Red banana and Green-red clones" was conducted at the Department of Plant Biotechnology, Vellayani, Thiruvananthapuram during the period from 2004 to 2006 with an objective of contrasting the expression of genes (chalcone synthase, dihydroflavonol 4-reductase and UDP: glucose 3-oxy-glucosyl transferase) involved in anthocyanin pigmentation in Red banana and Green-red clones. Experiments were also conducted to differentiate the clones based on total sugar concentration and protein profile.

Reverse transcription-polymerase chain reaction was carried out to study the expression of genes. Heterologous primers designed based on the gene sequences of *Oryza sativa* and *Vitis vinifera* were used for RT-PCR. Total RNA from tissue culture shoot, leaves and fruit from three different stages of Red banana and Green-red clones were isolated using hot phenol method which gave an yield of 80 - 200 µg 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). The RT-PCR amplified products of the two clones, representing chalcone synthase (CHS), dihydroflavonol 4- reductase (DFR), and UDP: glucose flavonoid 3-oxy-glucosyl transferase (UFGT) were eluted and purified. The cDNA fragments were cloned to pCRII vector (TA Cloning Kit, Invitrogen. Inc., USA) and sequenced.

The nucleotide to nucleotide BLAST of cDNA clone of 183 bp from Red banana showed similarity with the chalcone synthase mRNA sequence of rice (Acc. No. X89859). cDNA from Green-red clone showed no significant similarity to any other chalcone synthase gene during homology search. The translated query *vs.* protein database (blastx) search exhibited similarity with shikimate 3- dehydrogenase, an enzyme in the biochemical pathway for aromatic amino acids. The cDNAs synthesized

from Red banana and Green-red clone with gene specific primers for dihydroflavonol 4-reductase were having 354 bp and 325 bp length respectively. The homology search (blastn) revealed no similarities with any of the nucleotide sequences specific for dihydroflavonol 4- reductase. The cDNAs amplified from Red banana and Green-red clone with UDP: glucose 3-oxy-glucosyltransferase specific primers were of 361 bp and 345 bp, respectively. The homology search using blastn showed similarity with mRNA sequence for UDP: glucose 3-oxy-glucosyltransferase in grapes. In Red banana the blastx search revealed similarity with a glucosyltransferase from *Synechocystis* sp. There was no similarity for UDP: glucose 3-oxy-glucosyltransferase cDNA of Green-red clone in the NCBI database.

SDS-PAGE analysis showed no difference in the protein profile of Red banana and Green-red clone. Total sugar content in the peel, pulp, peel together with the pulp of red banana and Green-red clone did not showed any significant difference.

The expression analysis of the key genes of the general pathway showed no significant difference in both the clones. All the three genes selected for the study were present in both Red and Green-red clones. The genes isolated were not totally identical in the two banana clones. Further studies are needed to get a better insight into the cause of colour change.