CLONING AND EXPRESSION OF FLAVONOID 3'5' HYDROXYLASE PIGMENT GENE FOR DEVELOPING ECO-FRIENDLY COLOUR COTTON (*Gossypium* spp.)

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I. INTRODUCTION

Cotton (*Gossypium* spp.) has made profound influence on civilization by distinguishing the every existence of human being from other animals. Cotton in the form of cloth suffices one of the basic necessities of the human population. To a farmer, cotton is a commercial crop. Contribution of cotton to our economy is of high significance. India has the largest area under cotton (8.5 m ha) in the world occupying third position in production. The annual Indian cotton production is 232 lakh bales with the productivity of 443 kg per ha. India contributes 9 to 10 per cent of the cotton to the cotton basket of the world.

Almost all commercial cotton in the world has white lint though there are genotypes / species which produce naturally coloured cotton and most of the wild species of cotton have coloured lint or fuzz. Though historical evidences like the fossils obtained from the excavations at Huca Preita in Northern coastal Peru indicated the usage and cultivation of colour cottons with lint colour varying from tan to red shades before 2500 B.C., only some of which exist today. It seems others have been lost, as they have never been described in the botanical literature (Stephan, 1975 and Apodaca, 1990). The ability of the white cotton to take up any colour to produce a large range of shades and colours in fabrics has led to the popularization of white cotton. Efforts and resources have been diverted to the improvement of yield and fiber properties suitable for spinning. In the journey from fiber to fabric of white cotton, large quantity of water and hazardous chemical dyes, which pollute the environment and affect the human health are used and released. The effluent from dying industry are often let in rivers and lakes making water unsuitable for consumption. Also there are reports of allergic reactions to the synthetic and /or dyed fabric.

In the recent past, the thinking of the other side of industrial revolution has made man to realize that there is an urgent need to protect the environment. This eco-awareness has led to the revival of colour linted cottons, which can do away the processes of dyeing and processing. These colour cottons have once again crawled out with the efforts of an American Scientist, Sally Fox who began to improve colour cotton since 1982 and has developed Naturally Colour Cotton Incorporation that markets colour cotton fabrics (Fox, 1987).

Conventional breeding strategies for the improvement of the two naturally colored cotton in other countries and back in India have proved without doubt the suitability of brown and green cottons for the fabric production (Khadi *et al.,* 1998; Fox, 1987). The commercial sale of these garments have fetched upto three times higher prices than dyed fabrics as they are devoid of carcinogenic dyes. Fifty per cent of the cost of production of a fabric is towards the dye cost and processing. Naturally pigmented cottons not only are economical as they can do away with these but also eco-friendly as they can avoid the use of carcinogenic dye chemicals in the fabric and also minimize the effluent of the dyeing industries.

Reports also indicate that these cottons can be a remedy to psychosomatic diseases. In the present era of serious concern of the consequences of the depleting Ozone layer which protects the earth from of the UV rays which have been identified as the cause of an increased incidence of skin cancer especially in the European population, a recent finding shows the pivotal role of naturally pigmented cottons can play in the human health.

On one hand, the knowledge of all these desirable features of the naturally pigmented cottons, there is no second thought for the adaptation of these cottons, on the other hand history shows that though they co-existed with white cotton and they were left behind in the mid way. The main reason for this is the existence of only a few shades in naturally pigmented cottons while white cottons could take up any dye to give a wide range of colours and shades to satiate the needs of the textile industry. Therefore diversification of the lint colour would have a major impact on making the cultivation of naturally pigmented cottons a commercially viable venture. India with its thousands of years of cotton growing experience, rich genetic resources and textile industry is well poised to take up the challenge of developing coloured cottons for commercial cultivation.

The developments in the field of biotechnology, specifically recombinant DNA technology has made an impact to agriculture. The development and commercialization of transgenic crops has opened up newer avenues and ventures to the researchers to aim at.

The colouration of flowers and fruits is due to the accumulation of flavonoids (including anthocyanins), carotenoids and betalanins. Anthocyanins are major flower pigments in higher plants and have been studied extensively. The biochemistry and enzymology of the anthocyanin pathway is well understood and virtually all the genes that encode the enzymes of biosynthesis have been isolated (Holton and Cornish, 1995).

The naturally pigmented cottons available in nature have very few colours and mostly only brown and green and do not have blue shades. Efforts can be initiated to exploit the transgenic technology used in floral industry for the development of a colour / shade in cotton lint different from the already existing ones.

Several important new genes required for flavonoid biosynthesis have been characterized in a variety of plant species over the past few years, including some with direct practical applications. Flavonoid biotechnology has become a powerful tool to manipulate flower color in horticulture industry. .

In the world of blooms, blue is particularly difficult hue to achieve. The blueness seen in flowers like petunia depends on the synthesis of a pigment delphidin by flavonoid 3'5' hydroxylase (F3'5'H). The biotechnology company Florigene in Australia was founded to pursue the development of blue rose, carnation and chrysanthemums and so far has developed four varieties of transgenic “blue” carnation by transferring the viola F3'5'H gene which appear mauve than truly blue.

Therefore the first of its kind of attempt to isolate the flavonoid 3'5' hydroxylase gene, responsible for the main enzyme for the blue hue in flowers to transfer it into cotton with the following objectives was done.

1. Cloning of Flavonoid 3'5' Hydroxylase pigment gene from petunia and sequencing
2. Cloning F3'5' H gene in a plant expression vector and transformation into *Agrobacterium tumefacians*
3. Transformation of cotton with F3'5'H gene to study the expression

II. REVIEW OF LITERATURE

Naturally coloured cotton has its origin in the ancient America, where weavers cultivated and spun their native green and brown coloured cottons since their domestication about 4,500 years ago (Narayanan and Sundaram, 1996). Most of the naturally pigmented cotton lines grown commercially in the world have descended from pre-Columbian stocks selected by ancient Americans (Stephens, 1975). Early farming societies independently selected, domesticated and improved two entirely different tropical, perennial species of cotton, which had very poor fibre properties along with sparce fibres. Today improved cultivars with high yield, day length neutrality and annualized with easily ginned abundant fibre of uniform colour dominate the naturally pigmented cotton picture. The source of these improved genotypes can be traced back to 5000 years both cytogenetically and archeologically to the cultural centers of Central America and the Andean coast of western South America.

2.1 Colour cotton in world

Evidences indicate the cultivation of coloured cotton for indigenous and commercial use in Guatemala, Mexico, Colombia, Peru, Haiti, China, Egypt, India and Russia during 1800's. Some twelve shades of natural brown cotton were identified in Northern Peru at the end of the last century. Since 1982, the Native Cotton Development Project of Peru has recovered a number of landraces of naturally pigmented cotton, now commercially marketed by Pakucho Pax. Pakucho Pax Co-ordinates and markets the naturally coloured cotton produced by hundreds of small growers and also maintains some 75 different landraces of white and naturally pigmented fibre lines. The estimated 15,000 peasants and Indians who till today cultivate coloured cotton in Peru is the largest single group of naturally coloured lint producers worldwide. Six principal colour lines have been recovered and stabilized by project researchers viz., cream, tan, medium brown, reddish brown, chocolate brown and mauve (Singh *et al*., 1993).

2.1.1 North America

Naturally coloured cotton has been cultivated since 1604. The publicity given to the Soviet brown cotton production by the American press during World War II led to spinning tests on Nankeen brown and Arkansas green. In California, Gus Hyer, an USDA cotton geneticist, worked with coloured lint lines largely of upland varieties for several decades. Hayer's seed stocks have formed the foundation and improvement that began in California in the 1980's. Sally Fox, a plant breeder in Arizona and California working since 1982 using traditional techniques has developed several unique varieties which have been granted plant variety protection status (Fox,1987). The lint of these varieties have been commercially marketed as Fox Fibre ® since 1989 and Natural Cotton Colours, Inc has been formed in Arizona which is privately held.

2.1.2 Russia

The deficit in the supply of petroleum-based dyes during the Second World War, Soviet textile engineers creatively resorted to the use of these naturally pigmented fibres to add colour to the otherwise undyed cotton fabrics. During World War II USSR had put for sale about 700 metric tons of naturally coloured cotton lint (Ware and Bennedict, 1962). A wide range of natural hues were under production in Russia. Russians have indicated three distinct colours "Sand fresh", "Red brown" and "Green brown". Russian workers have been working on coloured lines for the past thirty years during which they have developed some 200 different varieties of lint (Veerland, 1993; Khadi and Kulkarni, 1996).

2.1.3 China

Cotton Research Institute, Hebei maintains around 40 colour linted lines of which the fibre colour is good but quality of the fibre is poor with low fibre length and strength (Veerland, 1993).

2.1.4 Meso-America

Meso-America comprises of countries like Peru, Mexico, Guatemala, Brazil and other neighbouring countries. The domesticated and semi domesticated varieties of naturally coloured cotton belonging to *G. barbadense* have influenced socially, economically, ceremonial and political lives of indigenous people of millenia. Naturally coloured cottons are known by different names like *Algodon pais*, *coyoqui*, *coyuche*, *etc.* Archeological remains found in the excavations at Hauca Prieta on the North Peruvian coast indicates the cultivation and use of chocolate brown cottons belonging to *G. barbadense*. Deeply pigmented lint was recovered in most other coastal archeological sites in subsequent period (Birds, 1985). In the vicinity of these early archeological sites coloured cottons are still cultivated in the present farming and coastal fishing villages (Veerland, 1993). Fabric made by Andean weavers shows two distinct colours light brown and chocolate brown of which dark brown was used to make fishing nets (Veerland, 1993: Apodoca, 1993).

Several varieties of perennial and natural brown cotton known as *Cuyuscate* are still grown in lands of Guatemala and similarly in its highland brown cotton called as *ixcoco* is still spun in several communities (Veerland, 1993).

2.1.5 USA

Naturally coloured cotton was cultivated by subsistence farmers and by slaves on large plantation estates, which was spun and woven by hand. Naturally pigmented cotton was spun into attractive cloth and sold in many southern states of USA. Green and brown cotton yarn and fabric was produced for world war II in many parts of USA (Veerland, 1993; Apodoca, 1990; Narayan and Sundaram, 1996). During colonial period coloured forms like Chinese Nankin, Central American, Mexican and those from Peru were introduced in USA.

2.1.6 Egypt

Brown fuzz dated about 2500 BC was recovered from Afia in Egyptian Nubia. The material could not be assigned to both old world cotton or to the presently grown cotton in Egypt. Pigmentation in Egyptian cotton was first noted in the Ashmouri valley and a variety named after the valley was developed which had long, strong fibre with a golden brown colour and could be readily ginned from naked seeds. Hand spinners intensively exploited it. Another variety Mit Afifi having rich, darker brown lint with 34 mm length was developed.

2.2 Colour cotton in India

From time immemorial coloured cottons are known in diploid cottons and were in cultivation in Asia, particularly in Indian subcontinent, China, Central Asian republics of former Soviet Union *etc.* In *Ayurveda charithrum* there is a reference to development of red and blue lint using different plant products. In Indian subcontinent coloured cottons like brown, khaki and red were grown in specific locations over large areas. They were commercially used in hand spinning, weaving novelty fabrics and for domestic use. In Assam non-*cernum* cottons with black, brown khaki and creamy white linted types were found. In Kumpta cottons grown in Karnataka red tinged types were available. In Bengal world famous Dacca muslins and high-count hand spun fabric was made from white as well as red *G. arboreum* (Narayan and Sundaram, 1996). In Tripura soft brown types are grown and used into weaving wrape stripe pattern fabric and gains given importance as it is related to religious and social customs (Paul, 1989).

In coastal Andhra Pradesh, brown linted cotton varieties like Red Northerns, Cocanadas-1 and Cocanadas-2 were grown during first half of the present century in about 25,000 ha area. These were spun and woven into fabric and some quantity was exported. Still in areas of Tripura (Paul, 1989) and tribal areas of Andra Pradesh (Kakinada area) brown cotton is grown for domestic use. Especially in Kakinada area around 10,000 farmers are engaged in cultivation and use of coloured cottons (Basu, 1995).

Extensive efforts were done to develop naturally coloured cottons for commercial cultivation by scientists of Agricultural Research Station, University of Agricultural Sciences, Dharwad, India, has resulted in a large number of genotypes of both in *G. hirsutum* and *G. arboreum* with various stable shades of brown ranging right from off white to dark brown. DDCC-1, an almond coloured variety belonging to *G. arboreum* has been released which is on par with the commercial white *desi* variety, Jayadhar grown in the Northern belt of Karnataka. It has been tested for the manufacture of fabrics and stability of colour. The Karnataka Village Industries Commission (KVIC) and Karnataka State Khadi Gramodyog (KSKG) have come forward for the commercial cultivation of DDCC-1 on contract farming.

2.3 Importance of naturally coloured cottons

Organic agriculture is an ecological production management system that promotes and enhances biodiversity, biological cycles and soil biological activity. It is based on minimal use of farm inputs and on management practices that restore, maintain and enhance ecological harmony.

Though the coloured cotton has created a growing niche market in the developed countries, its fibres in general are too short and weak to be spun. Hence, the varieties and hybrids with the pigmented lint with acceptable fibre qualities are being developed to give a fillip to agriculture, trade and industries (Khadi and Kulkarni 1996; Amuda, 1994).

The growing concern for environment and health hazards associated with the use of synthetic dyes, particularly in western countries have given fillip for cultivation of naturally coloured cotton and research in our country (Basu, 1995). A revival of interest in chemically free raw materials, minimally handled, environmentally “friendly” production strategies and transformational processes are contributing to a new demand for naturally pigmented cotton fibre (Veerland, 1993).

White cotton has to be bleached and processed before dyeing with colours. Almost all these processes involve the use of chemicals, which are known to cause health hazards, for example chlorinated products, bleaching agents, phenols, formaldehyde which cause skin disease.

Dyes containing traces of heavy elements such as arsenic, lead, cadmium cobalt, zinc and chromium are skin irritants, especially for children. Azodyes are proven carcinogens. The water from mills discharging into the water source pollutes the water and affect the aquatic life. Therefore many of the developed countries have banned the use of these hazardous chemicals and even the import of textiles dyed with these chemicals. Germany has banned the import of cloth dyed with azodyes. In a situation like this if not replace, naturally coloured cottons can initiate a humble beginning in the struggle to reduce the environmental pollution. Also naturally coloured cottons bring medical remedy for over fifty different somatic and psychosomatic diseases of man (Veerland, 1993). Previous studies along with the advantage of inherent color (Kimmel and Day, 2001) have also shown the flame resistance of brown cotton (Williams, 1994) and colour change (darkening instead of fading) occurring with certain laundering methods (Oktem *et al.,* 2003; Van Zandt, 1994). Various other studies indicate that colour fastness to light and washing is much better than the original fibre colours (Sundarmurthy *et al*., 1994). A study by Khyadi and Naik (1999) indicated that the fabric of naturally coloured cotton was darker by 131, 147 and 277 per cent after 20 washes with natural cleansing agents, soaps and detergents, respectively. Commercial scouring and mercerization increased the fabric colour by 338 and 440 per cent, respectively. A wide range of shades from parent colours like brown, green and inter mixing can be got. In extending the

boundaries of application, fibres of natural coloured cottons can be blended with polyester fibres, silk *etc*.

The study by Gwendolyn and Patricia (2005) demonstrates that naturally pigmented cottons have excellent sun protection properties (high UV protection factor (UPF), which are far superior to conventional bleached or unbleaded cotton (green cotton UPF = 30 to 50 +; tan UPF= 20 to 45 ; brown UPF = 40 to 50+; bleached conventional UPF = 4; unbleached conventional UPF = 8). The UPF values of the naturally – pigmented cottons remained high enough, even after 80 AFUs (American Association of Textile Chemicals and colorists fading units) of xenon light exposure, so that the fabrics merited sun protection ratings of "good" to "very good" according to ASTM (American Society for Testing Materials) 6603 voluntary labeling guide terms for UV-protection textiles. According to which an UPF rating of 15 and above is required before a fabric may be labeled sun protective.

2.4 Flavonoids

Flavonoids, including anthocyanins are ubiquitous compounds constituting about 5-10 per cent of the known secondary metabolites imparting vivid floral, seed and foliage colour in plants ranging from bryophytes to angiosperms. A large number of more than 6400 different flavonoid compounds are obtained in plants characterized by their wide occurrence and complex diversity. Flavonoids have many functions viz., colour and UV-B screening pigment, pollen fertility factors, signal molecules in plant microbe symbiotic associations, free radical scavengers, antifeedants, phytoalexins, plant defense response, cold stress response and modulators of hormone response (Madhuri and Arjula Reddy, 1999; Stefan *et al.,* 2003). Besides these biochemical, physiological and ecological functions many flavonoids play a health protecting role in human-diet and by this gave useful applications in the manufacture of foods, industrial products and biopharmaceuticals(Harborne and Williams, 2000). Plants are specialized in synthesizing and accumulating only a certain combinations of flavonoid compounds out of a large pool of known flavonoids. Such specificity at the species, genus and family level implies an evolutionary function associated with plant life.

The flavonoid chemistry began long ago and by 1970's the comparative chemistry of flavonoids had been well established. NMR and mass spectral analysis were extensively used to confirm the structural identity of flavonoids. Advances in genetic analysis of the flavonoid biosynthesis in plants were possible due to the naturally occurring, non-lethal and visually scorable distinct flavonoid mutant phenotypes serving as 'ready-made' genetic variability (Dooner and Robins, 1991).

In recent years, much effort has been directed at elucidating the flavonoid biosynthesis pathway from a molecular genetic point of view. Maize, Snapdragon (*Antirhinum major*) and Petunia were established as the first major experimental models in this system and work in these species led to the isolation of many flavonoid structural and regulatory genes (Holton and Cornish, 1995; Mol *et al.,* 1998). Spatial and/or temporal expressions of structural genes or enzymes of flavonoid biosynthesis in flowers, leaves and seedlings have also been well studied in many plants such as petunia (Brugliera *et al.,* 1994), Snapdragon (Jackson *et al.,* 1992), gerbera (Helariutta *et al.,* 1993), carnation (Stich *et al.,* 1992), rose (Tanaka *et al.,* 1995), egg plant (Toguri *et al.,* 1993), Arabidopsis (Pelletier *et al.,* 1997), grape (Boss *et al.,* 1996), Perilla (Gong *et al.,* 1997) and lisianthus (Nielsen and Podivinsky, 1997). Transcription of these genes is spatially and developmentally regulated in a well co-ordinated way paralleling flavonoid synthesis (Tanaka *et al.,* 1998). Structural and also several regulatory genes have been cloned, characterized and used in gene transformation experiments to modify flavonoid synthesis in specific plant tissues.

The diverse functions of flavonoid compounds in plants and in humans offers many potential and attractive targets for metabolic engineering approaches. Generally, three basic strategies would be used for flavonoid pathway modulation in specific plant tissues,

- Expression of novel sense derivatives of genes not present in the gene pool of the target plant to open the pathway to new metabolites.

- Expression of sense derivatives of a suitable gene to open the pathway to new metabolites by overcoming genetic blocks or rate limiting steps.
- Gene silencing to down regulate synthesis of undesired flavonoids.

2.4.1 Metabolic engineering of flavonoid pathway

In the first successful metabolic engineering experiments of the flavonoid pathway in plants, the maize A1 gene encoding dihydroflavonol 4-reductase (DFR) was introduced in a chemogenetically characterized mutant line of *Petunia hybrida* accumulating kaempferol(Km) and dihydrokaempferol (DHK) in flowers giving rise to a new orange *Petunia* phenotype not found in this species (Forkmann and Rahnau, 1987; Meyer *et al.,* 1987).

One of the most important functions of flavonoids is their contribution to the colouration of flowers, which attract the pollinators and other tissues. Since the experiments of Gregor Mendel in the 19^{th} century scientists have recognized the potential of plant and flower pigmentation as a tool for elucidating some of the basic principles of genetics and biochemistry. Because of the commercial value of flowers, their pigmentation has also been a subject of applied research for nearly four centuries. Anthocyanins are coloured class of flavonoids and accumulate in vacuoles. There are six major anthocyanidins (Chromophores of anthocyanins) and their modification with glycosylation and acylation results in a wide variety of anthocyanins. Besides the structure of anthocyanin, changes of vacuolar pH, inter molecular stacking (self-association of anthocyanins and co-pigmentation of anthocyanins with polyphenols), intramolecular stacking of aromatically modified anthocyanins, metal complexation and cell shapes give almost infinite flower colours (Goto, 1987; Goto and Kondo 1991; Brovillard and Dangles 1994; Mol *et al.,* 1998).

P450 genes are recalcitrant to purification due to unstability, low abundance and membrane bound nature of the enzyme. T-DNA tagging of the mutant allel *fahl* mutant of *Arabidopsis* was used to define a F3H (ferulate – 5 hydroxylase) to a new family of cytochrome P450 – dependent mono oxygenases designated as CYP84 (Knut *et al.,* 1996).

As many structural genes and regulators of anthocyanin biosynthesis have been isolated from Petunia, candidate gene analysis of anthocyanin pigmentation loci in other solanaceae crop species like tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum*), pepper (*Capsicum* spp) and egg plant (*S.melongena*) was done to identify candidate genes that may correspond to loci of natural colour variation, B anthocyanin – related genes were localized on a tomato F_2 genetic map. Gene map positions were then compared to mapped mutants in tomato and through comparative genetic maps to natural variants in potato, egg plant, and pepper. Similar map positions suggest that the tomato mutants *anthocyaninless, entirely anthocyaninless* and *anthocyanin gainer* correspond to flavonoid 3'5' hydroxyalase, anthocyanidin synthase and the Petunia *Myb* domain transcriptional regulatory gene *an2*, respectively. Similarly potato R, required for the production of red pelar gonidin – based pigments, P, required for production of purple delphinidin – based pigments and I, required for tissue – specific expression in tube skin appear to correspond to dihydroflavanol 4-reductase, F3'5'H gene and *an*2, respectively. The map location of an2 also overlaps pepper 4 and eggplant *Fap*10.1, *11a*.10.1 *Ira* 10.1, *va.* 10.1, *pa*.10.1 and *ca* 10.1, suggesting that a homologue regulatory locus has been subjected to parallel selection in the domestication of many solanaceae crops (Jong *et al.,* 2003).

Tomato fruit contain only in their peel small amounts of flavonoids, mainly naringenin chalcone and the flavonol rutin, a quercetin glycoside. To increase flavonoid levels in tomato as they are potent antioxidants and also correlated to reduce cardiovascular diseases, maize transcription factor genes LC and CI were expressed in the genetically modified tomato fruits. Expression of both these genes was required and sufficient to upregulate the flavonoid pathway in tomato fruit flesh, a tissue that normally does not produce any flavonoids (Arnaud Bovy *et al.,* 2002). In Gerbera hybrids, flavone synthesis is controlled by the locus *Fns*. The responsible enzyme, flavone synthase II, belongs to cytochrome P450 monooxygenases. From two different chemogenetic defined Gerbera lines with dominant (*fns +*) or recessive

(*fns fns*) alleles at the locus *Fns,* a cytochrome P450 fragment (Cyp DDd 7a) was isolated using a differential display technique with upstream primer based on conserved heme – binding region of P450 protein. The full-length cDNA (CYP 93B2), which contained the open – reading frame and part of CypDDd7a sequence, was isolated via 5' RACE and end-to-end PCR with gene specific primers. Northern blot analysis of total RNA indicated that the CYP 93B2 gene was only transcribed in lines with dominant allele (*fns*) and that the transcript levels during flower development were in agreement with enzyme activity of FNS II and flavone accumulation (Elomaa *et al.,* 1993).

Blue and violet flowers contain derivatives of delphinidin; red and pink flowers derivatives of cyanidin or pelargonidin. Differences in hydroxylation patterns of these three major classes of anthocyanidins are controlled by the cytochrome P450 enzymes, flavonoid 3' hydroxylase, (F3'H) and flavonoid 3' 5' hydroxylase (F3'5'H).

Anthocyanins are glycosylated derivatives of anthocyanidins, which differ in the degree of hydroxylation and subsequent methylation in the B-ring. The addition of extra hydroxyl groups to the anthocyanin B-ring generally leads to a blueing of flower colour. Several soluble and microsomal enzymes control anthocyanin biosynthesis. Genes encoding many of the soluble enzymes have been cloned by transposon tagging, differential screening and screening with antibodies raised against purified proteins. But genes encoding the microsomal enzymes F 3'H and F 3'5'H have eluded these cloning strategies. F 3'5'H activity was first detected in microsomal preparations from flower of Verbena, which contain the anthocyanins based on delphidin and later in petunia.

The F3'5'H is a microsomal P450 enzyme. Cytochrome P450's are heme containing enzymes widely found both in prokaryotes and eukaryotes. Seventy families and over a hundred subfamilies of P450 genes have been isolated from prokaryotes, fungi, animals and plants (Nelson *et al.,* 1996). It has been difficult to purify the P450 enzymes for further enzymological studies and amino acid sequencing and therefore the first plant P450 with known function was cloned only in 1993. To isolate F 3'5'H clones from petunia petal cDNA library PCR based strategy using degenerate oligonucleotide primers designed to conserved P450 heme binding domain was used (Holton *et al.,* 1993). The petunia mutant line devoid of F 3'5'H was transformed with the cloned gene showed complementation of F3'5'H mutations in petunia. The petunia genomic F3'5'H was submitted by Brugliera *et al.* (1999) and Hwang (2002).

Florigene Ltd. (Australia) and Suntory Ltd. successfully developed transgenic violet carnations by introducing petunia F3'5'H and DFR genes into a DFR deficient white carnation. The transgenic violet carnations named Moondust have been marketed in Australia and became the first transgenic floricultural crop. Darker versions, Moon Shadow have also been obtained (Tanaka *et al.,* 1998).

A full length cDNA, TG1 was isolated from *Prairie gentian* by heterologous hybridization with a petunia cDNA, AK14, which encodes F 3'5'H. The cloned TG1 and AK14 were transferred to tobacco and petunia mutant (lacking F3'5'H) which had dramatic change in the flower colour from pink to magenta with a high content of 3'5' hydroxylated anthocyanins (Shimada *et al.,* 1999). However, it has also been later reported that an additional petunia gene, a cytochrome b5 is required for full activity of F3'5'H (Vetten *et al.,* 1999). Putative F3'5'H has been cloned from several species *viz*., *Solanum tuberosum, Solanum melongena, Vinca major, Eusroma grandiflorum etc.* however, the complete gene and its expression studies are very limited.

2.5 Transformation of cotton

The past two decades have seen major advances in plant transformation, and a wide range of species can now be genetically transformed (Christou, 1995; Siemens and Schieder, 1996; Tarek *et al.,* 2002). These technologies have had considerable impact both on basic scientific research, where they have enabled advances in understanding plant processes and on production of economically viable agricultural products like transgenic plants.

2.5.1 *In vitro* transformation

Although many different techniques have been tested for gene delivery to plant cells, *Agrobacterium* mediated transformation has been extensively employed. The first transgenic plant of *Nicotinana tobaccum* was produced via *Agrobacterium* mediated transformation (Horsch *et al.,* 1984). With this success many crop plants were transformed via *Agrobacterium*. This is the simplest method now available for transferring genes into intact plant tissue.

Potrykus (1990) reported that shoot apical meristem of plant generates the whole plant at higher frequency. Gene transfer to meristem cells could therefore, overcome many of the regeneration problems of tissue culture systems in many important crop species.

Medford (1992) reported that meristem is often confused with the complete shoot apex, which also contains the leaf primordia and the young leaves. Further more, the meristem as a tissue may represent a complicated pattern of cells. Each of these cells may differ physiologically due to its unique position in the meristem.

Dillen *et al.* (1997) indicated that temperature plays an important role in transformation with *Agrobacterium tumefaciens.* In their results, the best transformation efficiency was obtained at 22°C in both *Phaseolus acutifolius* callus and tobacco leaves, irrespective of the type of helper plasmids. Although *in vitro* co-cultivation is normally done at 25°C, they showed that a lower temperature (19-22°C) was more optimal because in *planta* tumour formation occurred more frequently at 22°C.

Gould *et al.* (1998) presented a protocol for rapid genotype independent transformation and regeneration of cotton (*Gossypium* spp.) from shoots isolated form germinating seedlings. They inoculated the isolated shoots with a super virulent strain of *Agrobacterium tumefaciens* and subjected them to a mild antibiotic selection and directly regenerated as shoots *in vitro.* By this method the shoots did not dedifferentiate and mutation rates were low. Rooted shoots could be obtained within 6-10 weeks of isolation and inoculation depending on the cotton cultivar.

Hemphill *et al* (1998) reported a clonal propagation system to regenerate mature cotton (*G.hirsutum* L.) plants from pre-existing meristem from *in vitro* grown tissues. Shoot apices, lateral nodes and cotyledonary nodes were co-cultivated with *Agrobacterium tumefacieus* and grew to a two-leaf stage by this *in vitro* culture system. They further reported that this procedure resulted in 121 kanamycin selected shoots and 40 mature viable plants, which produced viable T_1 seeds. Mature T_1 plants expressed GUS activity in pollen grains that suggested that the transgene was inherited by progeny.

Wang *et al.* (1998) have successfully done transformation of four upland cotton cultivars via *Agrobacterium* mediated transformation. Hypocotyl segments from asceptic seedling were used as transgene recipients. After co-cultivation, kanamycin resistant calli were screened and somatic embryos and regenerated plants were obtained on various media. Transgenic cotton plants were confirmed by ELISA, PCR and southern analysis, and bioassays demonstrated that the transgenic plants had significant resistance to larva of cotton bollworm.

Zapata *et al.* (1999) reported that transgenic cotton (*G.hirsutum*) plants of Texas cultivar were obtained using *Agrobacterium* mediated transformation coupled with the use of shoot apex explants. Regeneration of primary plants was carried out in a medium containing 100 mg/l of kanamycin and the progeny obtained by selfing to plants were subjected to kanamycin screening. Surviving plants showed more than one copy of T-DNA. The use of shoot apex circumvents the problem of genotype dependent regeneration of cotton.

Satyavathi *et al.* (2002) have given a protocol for consistent production of transgenic cotton plants in three Indian varieties. Shoot tip explants were transformed by co-cultivation with *A. tumefaciens* strain LBA 4404. Among the different combinations of BAP and NAA tested, 0.1 mg/l of BAP and NAA in the medium influenced efficient regeneration of shoots by organogenesis. Shoot bud proliferation and elongation were achieved in 3-4 weeks time on medium supplemented with GA_3. The putatively transformed shoots were harvested and placed for rooting on medium containing IBA and 75 mg/l kanamycin. Transgenic plantlets were obtained in 12-16 weeks from the time of gene transfer to the establishment in pots.

2.5.2 *In planta* transformation

In most of cases, transgenic plants are produced by methods which include the transformation of individual plant cells followed by regeneration of whole plants from such cells (Christou, 1995; Fraley *et al.,* 1983; Potrykus, 1991). Although these approaches work well for some species, in others it has proven difficult to regenerate whole plants from tissues susceptible to transformation. So the efforts were made to develop protocols for *in planta* transformation where there is no need of *in vitro* regeneration. There are only a few species for which transformation systems without tissue culture based regeneration steps are available (Anthony *et al.,* 2000).

2.5.3 Transformation methods that avoid tissue culture

A number of laboratories have pursued plant transformation methods that avoid tissue culture. In many cases these methods have targeted meristems or other tissues that will ultimately give rise to gametes (Chee and Slighton, 1995; Birch, 1997). The same is true of popular tissue culture based transformation methods for corn, rice, wheat and soybean, which target young apical meristem for transformation (Birch, 1997).

In *Hibiscus sabdariffa,* Hooykaas and Schilperort (1992) reported that the rate of *in planta* transformation may suggest that there is a strong interaction between plant genotype and the strain.

For non tissue culture approaches, both *Agrobacterium* and tungsten particles have been used in a number of species to transform tissues or apical meristem cells that are subsequently allowed to grow into plants and produce seeds (Chee and Slighton, 1995; Birch, 1997). However, transformed sectors did not persist into gametes at reasonable frequencies or the methods were difficult to reproduce (Birch, 1997). Chowrira *et al.* (1995) reported that gene transfer through electroporation into intact meristems, *in planta,* was also possible.

Two rapid and simple *in planta* transformation methods have been developed for the model legume *Medicago truncatula* (Anthony *et al.,* 2000). The first approach involved infiltrating flowering plants with a suspension of *Agrobacterium.* The second method involved infiltration of young seedling with *Agrobacterium.* In both cases, a proportion of the progeny of the plants were transformed. The transformation frequency ranged from 4.7 to 76 per cent for the flower infiltration method and from 2.9 to 27.6 per cent for the seedling infiltration method. The transformed plants were genetically stable and the analysis of T_2 generation indicated that the transgenes were inherited in a Mendelian fashion. Both the procedures resulted in a mixture of independent transformants and sibling transformants. Transformation by infiltration of adult plants with *Agrobacterium* has also been reported for Pakchoi (Liu *et al.,* 1998) and other members of *Brassicaceae*.

In *Arachis hypogea,* Rohini and Rao (2000) reported direct transformation in 5 of out of 150 tested through embryonic axis after wounding. Similarly, Yaye *et al.* (2004) produced transgenic *Hibiscus sabdariffa* plants by using embryonic axes of mature seeds with one cotyledon excised and infected by immersion in a suspension of *Agrobacterium* LBA 4404 strain.

Ken Feldman and David Marks carried out early stages of the revolutionized transformation work with *Arabidopsis.* They applied *Agrobacterium* to *Arabidopsis* seeds, grew plants to maturity in absence of any selection, then collected progeny seeds and germinated them on antibiotic containing media to identify transformed plants (Feldmann and Marks, 1987; Feldmann, 1992). Although the procedure was difficult to reproduce consistently, successful rounds produced transformants at a high enough rate that thousands of transformed lines were produced in a matter a few years. These lines helped speed gene cloning by the *Arabidopsis* community (Azpiroz-Leehau and Feldmann, 1997).

Some of the laboratories have succeeded in generating transformed *Arabidopsis* lines by "clip'n squirt" methods (Chang *et al.,* 1994; Katavic *et al.,* 1994). Reproductive inflorescence were clipped off, *Agrobacterium* was applied to the center of the plant rosette, new inflorescence formed a few days later were again removed, *Agrobacterium* was reapplied, and plants were then allowed to develop and set seed. Transformants were obtained more reliably than with the seed treatment method, but the methods were only marginally more productive than traditional tissue culture approaches in *Arabidopsis* (Valvekens *et al.,* 1988).

III. MATERIAL AND METHODS

The present study was undertaken to clone the gene encoding Flavonoid 3'5' – Hydroxylase from native petunia with purple flowers and study its expression in cotton. The materials used and the methods employed are presented herewith.

3.1 DNA isolation from petunia variety with purple colour flowers

Genomic DNA from leaves of petunia genotype with purple flowers (Plate 1) was isolated according CTAB method (Saghai-Maroof *et al*., 1984) with minor modifications.

- One gram of young leaf sample of petunia was powdered in liquid nitrogen and fine white powder was transferred to 2ml sterile eppendorf centrifuge tube without thawing.
- 500 µl of extraction of buffer (Appendix I) was added and vortexed for 30 to 40 seconds.
- Then it was centrifuged at 4000 rpm for 10 minutes and the supernatant was discarded.
- 600 µl of lysis buffer (Appendix I) and 50 µl (5%) Sarcosyl was added and vortexed for 30-40 seconds.
- It was later incubated at 65°C for 1 hr by mixing thoroughly 2 to 3 times at an interval of 15 minutes.
- Equal volume of phenol: Chloroform: Isoamyl alcohol (IAA) (25:24:1) was added and mixed gently and centrifuged at 15000 rpm for 10 mins at 4°C.
- The supernatant was collected to which equal volume of chloroform: IAA (24:1) was added and mixed gently and centrifuged at 15,000 rpm for 10 minutes.
- The supernatant was transferred to fresh centrifuge tube to which 1/10 volume of 0.3M sodium acetate and double the volume pre-chilled ethanol was added and kept at room temperature for 30 mins.
- Centrifuged at 15,000 rpm for 10 mins at 4°C.
- The supernatant was discarded and the pellet was washed with 70 per cent alcohol.
- Pellet was dried completely and dissolved in 50 µl of T_{10} E1 buffer.
- Quantified according to ethidium bromide spotting method as described by Sambrook and Russel (2001) (Appendix II).

3.2 Primer designing and amplification of f3'5'h gene

Specific primers were designed using the DNA sequence information of F3'5'H gene deposited by Holton *et al.* (1993) (accession no. AF081575.1) with gene tool program (BTI Software). The primers were added with restriction sites of *Xba* and *Bam*H1 in forward and reverse primers respectively. Primers were got synthesized from IDT (Integrated DNA Technology) Inc. USA.

Primer Sequence:

Forward	5'-CGT CTA GAT CGG CCA TAT ACG TTT TCC TTT AGT – 3'
Reverse	5'-CGG GAT CCA CAA CCA ACA ACA TGC GCA ATT ATA G-3'

3.2.1. Amplification

A 10 pM primer concentration was used. XT-Taq polymerase enzyme and 10x assay buffer and individual dNTP's were obtained from M/S. Bangalore Genei Private Ltd., Bangalore. Eppendorf Master Cycler (5331) was used to run the PCR programme. The reaction mix required was prepared afresh to avoid handling errors and the master mix was distributed into different 0.5 ml centrifuge tubes and 2µl of template DNA was added. The PCR amplification was initially set at 5 gradient temperatures of primer annealing from 50 to 60° C and a temperature of 56° C was used for further studies.

Plate 1.Petunia genotype with purple flowers

PCR amplification

Stage	Step	Temperature	Duration	No. of Cycles
I	Initial denaturation	94	5 min.	1
II	Denaturation	94	1 min.	
	Annealing	56	30 sec.	15
	Extension	72	5 min.	
III	Denaturation	94	1 min.	
	Annealing	56	30 sec.	25
	Extension	72	5 min. + 20 sec extension	
IV	Final extension	72	30 min.	1
V	Hold	4	--	--

Table 1. Master mix for PCR (20 µl/tube)

Components	Quantity
10x assay buffer with $MgCl_2$	2 µl
dNTPs (10 mM)	2 µl
Forward primer (10 pM)	1 µl
Reverse primer (10 pM)	1 µl
XT-Taq DNA polymerase (5U/µl)	0.5 µl
$MgCl_2$ (3 mM)	1 µl
Template DNA (20 ng)	2 µl
Sterile double distilled water	10.5 µl
Total volume	20 µl

After completion of amplification, the samples were stored at 4°C untill further use.

3.2.2 Electrophoresis

Five µl of the amplified product along with 1 µl of loading mixture (Appendix IIa) was loaded onto 1.0per cent agarose gel (Appendix IIc) along with λ-DNA Hind III digest as DNA molecular weight marker. Electrophoresis was done at 50 V for initial 30 minutes and then 70 V for 1.5 hours. The buffer used was 1x TAE at pH 8.0 (Appendix IIb). After separation, the DNA bands in gels were visualized on a UV transilluminator and documented using a gel documentation system (Uvitec Cambridge, England).

3.3 PCR based cloning

3.3.1 Gel elution of the PCR amplicon

The specific sharp band of DNA amplicon corresponding to 2.9 kb region of the λ-DNA Hind III digest was separated out using a sharp sterile scalpel by keeping the gel on a low intensity UV transilluminator and collected in a sterile pre weighed 2.0 ml micro-centrifuge tube. The Stratagene-Gel extraction kit was used to elute the DNA from agarose block as described in the users manual.

The purified DNA was quantified by ethidium bromide spotting method as described by Sambrook and Russel 2001 (Appendix IIb).

3.3.2 Cloning of PCR product

The purified PCR fragment of 2.9 Kb was ligated to pTZ57R/T cloning vector (2868bp) as described in Ins T/A clone™ PCR product cloning kit (K1214) from MBI, Fermentas, USA. The circular plasmid with insert was used directly to transform *E.coli* X-L1 Blue cells.

3.3.3 Ligation

For ligation, an optimal molar ratio of 1:3 vector: insert was calculated as per the table given in Appendix III. The component of ligation mix (Appendix IV) were mixed in a 0.5 ml micro-centrifuge tube and was incubated at 22°C overnight in a circulating water bath. A control ligation reaction was done using control PCR fragment provided in the kit by adding components described below.

3.3 Preparation of competent cells

The competent cells of *E.coli* X-LI Blue were prepared as given in Sambrook and Russel (2001) manual.

3.4.1 Preparation of overnight culture

About 2 ml of Transform Aid™ C-medium was inoculated with *E.coli* X-LI Blue and incubated overnight at 37°C at 175 rpm. From this, 1/10th volume of the overnight culture was added to pre-warmed C-medium in a culture tube and incubated at 37°C for 2 hours.

3.4.2 Preparation of competent cells

Transform Aid™ T-solution was prepared by mixing equal volume of T-solution (A) and T-solution (B). After this 1.5ml of fresh culture of the X-L1 Blue cells was dispensed in 1.5 ml micro-centrifuge tube and spun at maximum speed for 1 min at room temperature. The pelleted cells were re-suspended in 300 µl of transform Aid™ T-solution and incubated on ice for 5 min. Cells were re-suspended in 120µl of transform Aid ™ T-solution and incubated on ice for 5 min.

3.4.3 Preparation of DNA for transformation

DNA was prepared for transformation by dispensing 1 µl of super coiled (100 pg) or 2.5 µl of ligation mixture (200 ng of vector DNA) in a fresh micro centrifuge tube and incubated on ice for 2 min. For transformation, 50 µl of the re-suspended competent cells were dispensed in tube containing DNA and incubated on ice for 5 min. Control transformation was carried out using 2 µl of super coiled plasmid DNA of pTZ57R. Cells were plated on pre-warmed LB-Ampicillin agar plates containing X-Gal IPTG (Appendix V) and incubated overnight at 37° C. The recombinant clones were identified by the blue/white assay. After incubation for 12 hours, the white colonies, which have recombinant vector, were picked up.

3.4.4 Confirmation of clones

The confirmation of the recombinant clones was done by quick clonal analyses as described here in. Overnight culture of white colonies was taken from Luria agar plate, which was incubated at 37° C. The growth was scraped and rubbed along the walls of 0.5 ml micro-centrifuge tube. The cells were mixed in a 100 µl of suspension containing 50 mM Tris + 3%SDS pH 12.5. The cells were physically disrupted and 100 µl phenol: chloroform (1:1) was added and kept on a gel rocker for 10 min. The suspension was later centrifuged at 10,000

rpm for 1 min. After centrifugation, the aqueous layer was transferred to fresh tube, 20 µl was loaded on 0.7 per cent (Appendix Va) agarose gel along with λ-DNA *Hind III* digest.

Further confirmation of the presence of cloned fragment was done by PCR amplification of clones with specific primers along with the total DNA of petunia plant and cloning vector, respectively, used as positive and negative controls in the PCR.

The confirmation was also done through comparative restriction analysis of the selected clones and the control vector to ensure the presence of insert with *Xba* I and *Bam* HI and visualizing the agarose gel on a UV transilluminator.

3.5 Sequencing of clone

The amplicon of about 2.9 Kb cloned in pTZ57R/T was sequenced using M13 primers employing gene walking technique at M/S. Bangalore Genei Pvt. Ltd., Bangalore. The sequence was subjected to analysis using BLAST algorithm available at http://www.ncbi.nlm.nih.gov.

3.5.1 Vector isolation

The alkaline lysis protocol of Brimbion and Dolly (1979) and Brimbion (1983) with certain modifications was used for isolation of the plasmids.

A 10 ml overnight culture was centrifuged at 4,000 rpm for 10 minutes at 4° C., the supernatant removed and the pellet was washed with STE buffer (0.25 volume of original culture). The pellet was re-suspended in 200 µl of ice-cold alkaline lysis solution I (Appendix VI) by vigorous vortexing and transferred to a micro centrifuge tube. Later, 400 µl of freshly prepared alkaline lysis solution II (Appendix VI) was added to each bacterial suspension and the contents mixed by inverting the tube rapidly five times and stored on ice. To this suspension, 300 µl of alkaline lysis solution III (Appendix VI) was added, the contents mixed by inverting the tubes several times, stored on ice for 5 minutes and centrifuged at 13,000 rpm for 5 minutes. Six hundred micro liters of supernatant was transferred to a fresh tube. For removal of protein in the supernatant, it was treated with equal volume of phenol chloroform and spun at 13,000 rpm for 2 min at 4° C. The aqueous layer was transferred to a fresh tube and 600 µl of isopropanol was added at room temperature. The contents were mixed and allowed to stand for 2 minutes at room temperature. Then, the solution was centrifuged at 13,000 rpm for 5 minutes at room temperature. The supernatant was discarded by gentle aspiration and the pellet was washed with 70 per cent ethanol and spun for 1 min at room temperature to recover nucleic acids. The supernatant was removed by gentle aspiration and the tube was stored at room temperature until ethanol evaporated. The pelleted nucleic acid was dissolved in 25 µl of T_{10} E_1 (pH 8.0) containing 20 mg per ml DNAse free RNAse A and the DNA solution was stored at – 20°C.

3.6 Cloning of the f3'5'h gene into plant transformation vector

For cloning of F3'5'H gene, plant expression vector, pHS100 obtained from Babha Atomic Research Center, Mumbai was used.

The vector was linearised with *Xba* I and *Bam* HI with buffer and BSA in the reaction using the following components. The complete restriction was confirmed by electrophoresis.

3.6.1. Gel elution of vector, pHS100

For elution of linearised vector, 1 per cent low melting point agarose gel was cast in cold room. The restricted sample was loaded along with 20x loading dye and electrophoresis was done for 3 hrs. in cold room using 10x TAE. Using sterile scalpel, the single sharp band

of 13 Kb was cut keeping the gel on low intensity UV transilluminator and collected in sterile 1.5 ml micro centrifuge tube. The tube containing gel block was incubated on 65°C water bath for melting. After complete melting, the tube was shifted to 42°C water bath for 30 min and the volume of molten agarose was measured. To 200 µl of molten agarose, 2 µl of agarase was added and the reaction was supplemented with 21 µl agarase buffer and incubated at 42°C for 1 hr. The tubes were immediately transferred into ice for 10 min for chilling and centrifuged at 13,000 rpm. The supernatant was taken in a fresh vial and treated with equal volume of phenol and centrifuged for 10 min at 13,000 rpm. To the supernatant, equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and spun at 13,000 rpm for 10 min. To the supernatant equal volume of isopropyl alcohol and 3M sodium acetate was added and incubated at – 20°C for 2 hrs. The tube was spun at 13,000 rpm for 10 min and the pellet was washed with 70 per cent alcohol followed by absolute alcohol. The pellet was air dried and dissolved in 10 µl sterile water.

3.6.2 Gel elution of the PCR amplicon

The single sharp band of DNA amplicon corresponding to the 2.9 kb region of the λ-DNA *Hind III* digest was eluted as described in section 3.3.1 and purified using Stratagene gel elution kit as per the instructions of user's manual.

3.6.3 Quantification of vector and insert

The purified vector DNA and insert DNA were quantified by ethidium bromide spotting method according to Sambrook and Russel 2001 (Appendix IIb).

3.6.4 Restriction of PCR amplicon

The eluted PCR amplicon was restricted with *Xba* I (partial digestion) and *Bam* HI (complete digestion) with buffer and BSA in the reaction (Appendix VIIa). The insert was purified using the Stratagene PCR purification kit and quantified. The vector pHS-100 also was restricted with *Xba* I and *Bam* H I completely.

3.6.5 Ligation

For ligation, an optimal molar ratio of 1:3 vector : insert was calculated as per the table given in Appendix IV. The components of ligation mixture was mixed into a 0.2 ml micro centrifuge tube and incubated (Appendix VIII) at 16° C overnight in Eppendorf Thermocycler.

3.6.6 Preparation of competent cells

Competent cells of *Ecoli* DH5α were prepared following the protocol of Sambrook and Russell (2001) with minor modification as described below.

Freshly saturated overnight culture was prepared by inoculating a loop full of fresh overnight culture of *E.coli* DH5α maintained on Luria agar plate onto 50 ml of Luria broth and was incubated at 37°C for 2 to 3 hours till the OD reached 0.4 at 600 nm. 25 ml of the culture was then distributed into 2 chilled centrifuge tubes of 50 ml capacity and incubated on ice for 30 minutes and was later spun at 6000 rpm for 7 minutes at 4° C. The supernatant was completely removed and the pellet was re-suspended in 12.5 ml ice-cold 0.1 M $CaCl_2$. The tubes were incubated on ice for 30 minutes and spun at 4000 rpm for 5 minutes at 4° C and the supernatant was drained off. The pellet was re-suspended in 1 ml of ice cold 0.1 M $CaCl_2$. These competent cells were stored at 4° C for 12 hours to increase the efficiency or they were stored in –80°C deep freezer by adding 100 µl of di-methyl sulfoxide (DMSO).

3.7 Transformation

For transformation of the ligated sample into DH5α host cells, the protocol of Sambrook and Russel (2001) with few modifications was followed.

100 µl of competent cells were dispensed into chilled 1.5 ml micro centrifuge tubes. The ligation mixture not more than 450 ng was added to competent cells and mixed gently. The mixture was stored on ice for 30 minutes and shifted to a preheated 42^{o} C water bath for 90 seconds and transferred onto ice to chill for 5 minutes. To this, 800 µl of Luria broth was added and kept on a shaker at 37^{o} C for 45 minutes to allow bacteria to recover and express the antibiotic marker encoded by plasmid. From this, 200µl of the sample was plated onto Luria agar plates containing kanamycin (50 µg/ml) and incubated at 37^{o} C for 12-16 hours.

3.8 Confirmation of clones

The isolated plasmid construct were restricted using *Xba* I and *Bam* H1 enzymes using buffer B. The restricted samples were loaded onto 1% agarose gel and electrophoresed along with λ-DNA *EcoR1/Hind III* digest as molecular weight marker. The clones were confirmed through the comparative analysis of plasmid and insert size with the marker.

3.9 Transformation to *agrobacterium*

For transformation of plant expression vector pHS-100 with the insert (F3'5'H) to *A.tumafaciens*, Triparental mating was followed. The culture used and their selection media are

- *E. coli* DH5α with vector – Donor (LB + Kan 50 mg/lit)
- pRK 2013 – Helper (LB+ Kan 50 mg/lit)
- *Agrobacterium* strain LBA 4404 – Recipient (YEP + Streptomycin 100 mg/lit + Rifamycin 25 mg/lt)

All the above three cultures were grown overnight in their respective selection media separately. One ml of culture of each of the three was transferred to 3 sterile 1.5 micro centrifuge tubes. They were centrifuged at 10,000 rpm for 5 minutes. The supernatant was thrown out and the pellet was washed with 0.01 $MgSO_4$. The centrifugation and washing with 0.01 $MgSO_4$ was repeated once more. Then the pellet was resuspended in 50 µl of fresh LB in all the three cultures. Donor, recipient and helper were mixed in two ratios of 1:1:1 and 2:1:2. Fifteen microlitre of the mixed ratios were separately spotted on plain YEP plates and incubated overnight at 28°C after patch mating. The overnight grown of culture was scraped with LB and streaked on YEP with Kanamycin50, streptomycin 100mg/l and Rifamycin 25mg/l.

3.10 *Agrobacterium* mediated transformation to cotton

3.10.1 Genotypes

Four cultivars of cotton *(Gossypium* spp), two brown pigmented and two white representing two species of cultivated cotton were used as follows.

- Sahana (*G. hirsutum*) White linted
- MB-25 (*G. hirsutum*) Brown linted
- PA-255 (*G. arboreum*) White linted
- DDCC-1 (*G.arboreum*) Almond linted

3.10.2 *In vitro* experiments

All the laboratory experiments were conducted under the well-defined conditions of the culture room maintained at 25± 2° C. Uniform light intensity (ca 1000/lux) was provided by fluorescent tubes over a light/dark cycle of 16/8 hours.

For all experiments borosilicate glass wares and analytical grade chemicals were used.

3.10.3 Methodology

3.10.3.1 Nutrient medium

Analytical grade Murashige and Skoog (1962) (Appendix IX) medium with 3 per cent sucrose, vitamins, 0.8 per cent agar supplied by Hi-media laboratories was used in this study. This medium was supplemented with different growth regulators at varying concentrations depending upon the purpose.

3.10.3.2 Preparation of explants

Seeds were surface sterilized with 70 per cent (w/v) ethanol followed by 0.1 per cent (w/v) aqueous mercuric chloride solution for 10 min and rinsed five times with sterile distilled water. Seeds were germinated under aseptic conditions on half strength MS. The pH of medium was adjusted to 5.8 prior to autoclaving at 121° C for 15 min.

3.10.3.3 Maintenance and growing of *Agrobacterium*

The *A.tumafaciens* LBA4404 strain containing pHS-100 was maintained on solid YEMA (Yeast Extract Mannitol Agar) medium containing 50 mg/l of kanamycin, 100mg/l streptomycin and 25 mg/l of rifamycin. Subculturing was done every month in fresh medium containing kanamycin and rifamycin. Single *Agrobacterium* colony was taken from the YEMA plate and was inoculated into 100 ml YEM liquid medium containing antibiotics and was incubated with shaking for 48 hours at 22° C and was used for transformation.

3.10.3.4 Transformation and plant regeneration

The transformation and regeneration techniques described by Zapata *et al*. (1999) with suitable modifications were employed.

3.10.3.5 Method of co-cultivation

Shoot tips with shoot apical meristem (SAM) were used as explants. Shoot tips of about 5 mm in length taken from 5 day old seedlings were used directly to co-cultivate with bacterial culture.

The bacterial culture was collected at late log phase (A_{600} 0.6). The explants were gently shaken in the bacterial suspension for about 15 minutes and were blotted dry on a sterile filter paper. Later they were transferred to the MS medium and co-cultivated under dark conditions for two days at 25± 2° C.

3.10.3.6 Culturing and sub culturing

After co-culture, the explants were washed with sterile distilled water, blotted dry and transferred to shoot induction medium (SIM) MS_1) containing 0.1 mg/l BAP and 400 mg/l cefotaxime.

Explants were washed with cefotaxime every 48 hours to remove the growth of bacteria. This was done 3-4 times depending upon the growth of bacteria.

After 20 days of keeping on shoot induction medium, the explants were transferred to shoot proliferation medium (MS_2) comprising MS_1 supplemented with 1.0 mg/l GA_3. On this medium they were allowed for 30 days for shot proliferation. Then the green healthy shoots were rooted in root induction medium (RIM) containing 0.3 mg/l IBA. (MS_3) for about 2 weeks.

3.10.4 *In planta* transformation

Seeds of all the four genotypes were sown in pots kept in controlled conditions in transgenic green house with 16 hrs. day photoperiod, 20^{o} C night and 25-30^{o} C day temperature. After seedling emergence and upto the expansion of two-cotyledonary leaf stage, the central meristem was sutured with sharp knife tip to about 3-4 mm, where the solid culture prepared was inoculated.

Solid Culture: A colony of bacteria grown for 48 hr. was taken from a petridish with the help of inoculation loop and was streaked on a fresh petriplate having solid YEMA medium with 100 mg/l streptomycin, 50 mg/l kanamycin and 25 mg/l rifamycin selections for pHS-100 and the plate was kept at 22^{o} C for 48 hr. Then the bacterial growth is collected in a sterile 5 ml borosil tube, mixed by vortexing and added with 100 µl of acetosyringone.

3.10.4.1 Selection of transformants

As the gene transferred is responsible for turning the pigment pathway toward shade like purple, megenta or blue hues is preceeded by a constitutive promoter CaMV35S, T_0 co-cultivated seedlings were observed for pigment colour changes for further confirmation with amplification of *npt* II gene and the F3'5'H gene.

IV. EXPERIMENTAL RESULTS

Results obtained on isolation, cloning and sequencing of the Flavonoid 3'5' Hydroxlase gene and transformation into cotton genotypes are presented below.

4.1 DNA isolation and amplification with specific primers

Total genomic DNA isolated from purple flowered petunia (Plate 1) leaf with specific primers at 5 gradient temperatures in the range 50 to 60 centigrade, with the taq DNA polymerase. There was specific amplification at all temperatures (Plate 2a). The amplicon size was about 2.90 kb. The ampliciation was done using XT-Taq DNA polymerase with proof reading activity in which very high specific and sharp amplication was observed (Plate 2b). This band was used for elution and cloning.

4.2 Cloning into pTZ57R vector and sequencing

The eluted PCR amplicon of 2.9 kb size was cloned using T/A cloning kit. The white colonies were the positive clones and were streaked to fresh plates with amphicilin selection. The plasmids isolated from positive clones were confirmed by the size, which was 5.7 kb as compared to 2.8 kb of pTZ57R/T. The plasmid was not restricted for clone confirmation as both the size of the insert and cloning vector cannot be visualized and eluted on agarose gel. Also for the same reason the insert from pTZ57R57 was not used for further cloning. The circular map of the pTZ57R/T vector along with the insert is shown in Fig. 1.

LEGEND

Plate 2a. Gradient amplification of petunia DNA

M : λ DNA/*Eco* RI + Hind III double digest

1 : Annealing temperature of 51.8℃

2 : Annealing temperature of 54.1℃

3 : Annealing temperature of 55.5℃

4 : Annealing temperature of 56.2℃

5 : Annealing temperature of 59.3℃

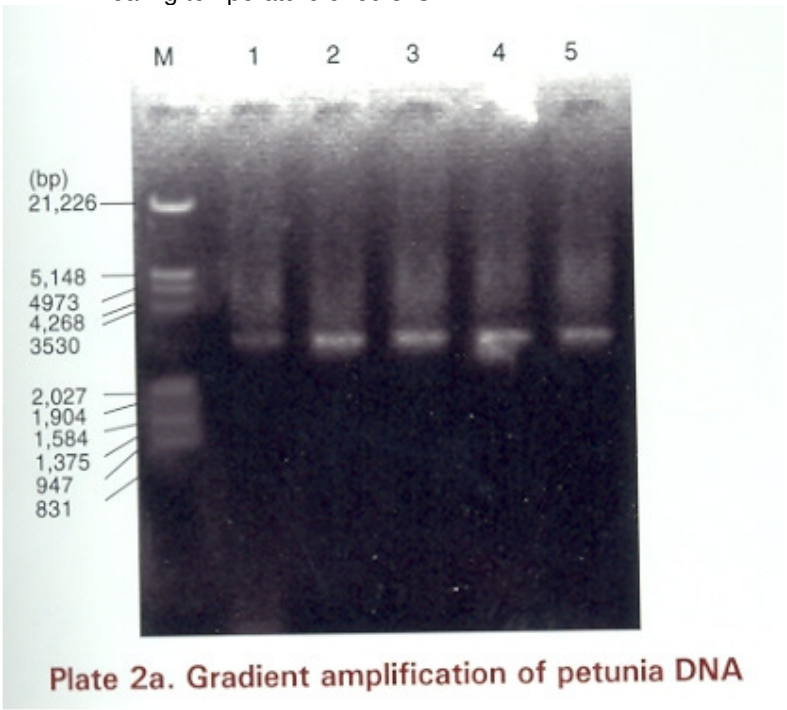


Plate 2a. Gradient amplication of petunia DNA

Plate 2b. Amplification of petunia DNA with XT-Taq and Taq polymerase enzyme

M : λ DNA/*Eco* RI + Hind III double digest

2 : Amplification of petunia DNA at 56℃ with XT-Taq polymerase

3 : Amplification of petunia DNA at 56℃ with Taq polymerase

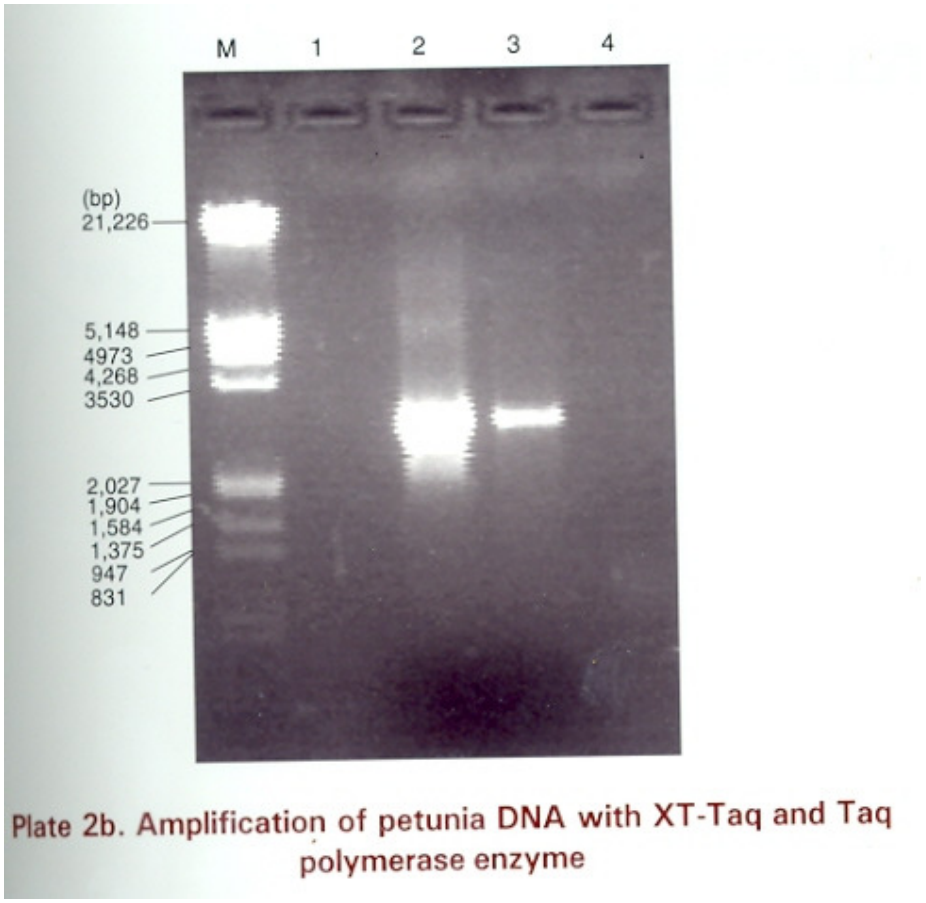


Plate 2b. Amplication of petunia DNA with XT-Taq and Taq polymerase enzyme

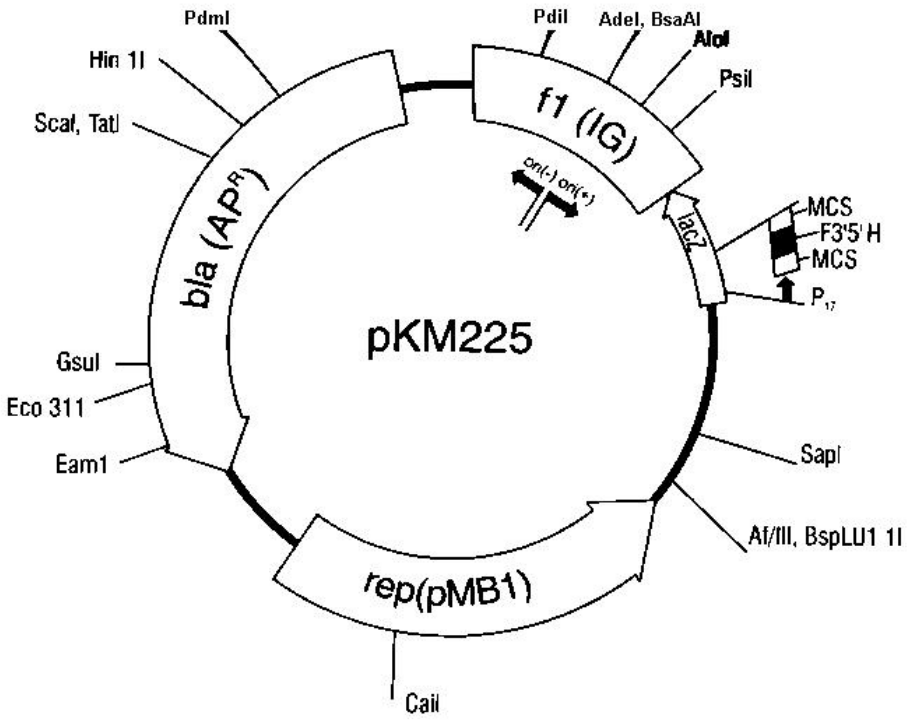


Fig. 1. Restriction map of the construct pKM225 containing full length F3'5'H gene in pTZ57R/T

Fig.1.Restriction map of the construct pKM225 containing full length F3'5'H gene in pTZ57R/T

The cloned fragment in clone pKM225 was sequenced by primer walking using M13 primer and the sequence is given in Fig. 2. The exact length of the insert was 2.936 kb. The start codon, the intron and exons were found by comparing the insert sequence of the present study and the genomic F3'5'H gene cloned by Hwang *et al.* (1998) (Accession No. AF081575). The three exon regions are shown in Fig. 2.

PCR analysis was done to confirm the pTZ57R/T clones which showed amplification of a fragment of 2.9 kb (Plate 3a). Restriction analysis for the enzymes was done with the help of Gene Tool and is given in Fig. 3. It was seen that the insert isolated for gene F3'5'H in this study had internal Xba I sites whereas the original gene sequenced which was used for primer designing did not have these sites. The presence of the restriction sites was further confirmed by restricting the pTZ57R clones with Xba I and *Bam*H1. The restriction products 1 Kb, 0.8 Kb and 0.5 Kb which were corresponding to the expected fragments of 1.05 Kb, 0.85 Kb and 0.58 Kb (Plate 3b).

The open reading frame (ORF) of the sequenced gene is given in Fig.4. The exons of the cds of the isolated sequence was translated to get the amino acid sequence (Fig. 5). It consisted of 475 amino acids .The presence of the internal Xba I sites had to be considered for cloning into plant expression vector.

4.3 Sequence analysis

The sequence was used to blast using the blast algorithm available at http://www.ncbi.nlm.nih.gov and the results are presented in Table 2. Among 187 hits the first 5 hits were to the petunia x hybrida Hf1 genes with 0.0 E values. The maximum homology was seen between the query sequence and the accession AF081575.1 Petunia x hybrida, flavonoid 3'5' hydroxylase cds (Fig. 6) and showed identifies of 2198/2211 and 650/651.

LEGEND

Plate 3a. PCR analysis of pTZ57R/T clones

1 and 2 : PCR amplification of petunia plant DNA

3 to 10 : PCR amplification of pTZ57R/T clones

M : λ DNA/*Eco* RI + Hind III double digest

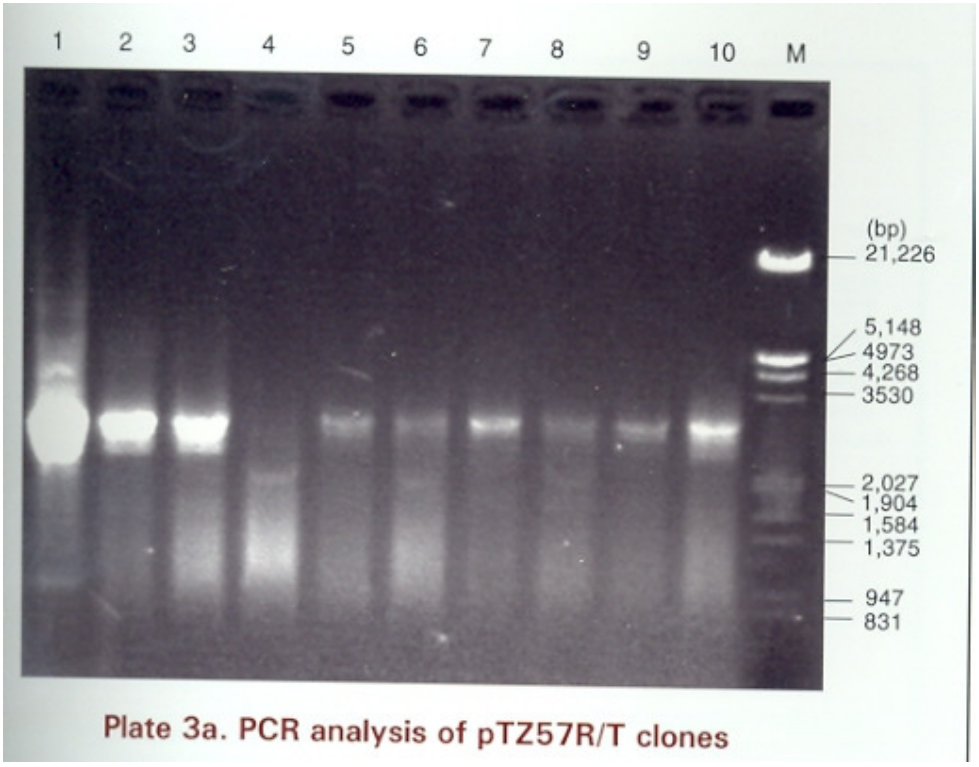


Plate 3a. PCR analysis of pTZ57R/T clones

LEGEND

Plate 3b. Restriction analysis of pTX57R/T clones with *Xba* I and *Bam* HI

1 and 8 : Restriction of pTZ57 R/T clones with *Xba* I and *Bam* H I

9 : Restriction of PCR amplicon with *Xba* I and *Bam* HI

M : λ DNA/*Eco* RI + Hind III double digest

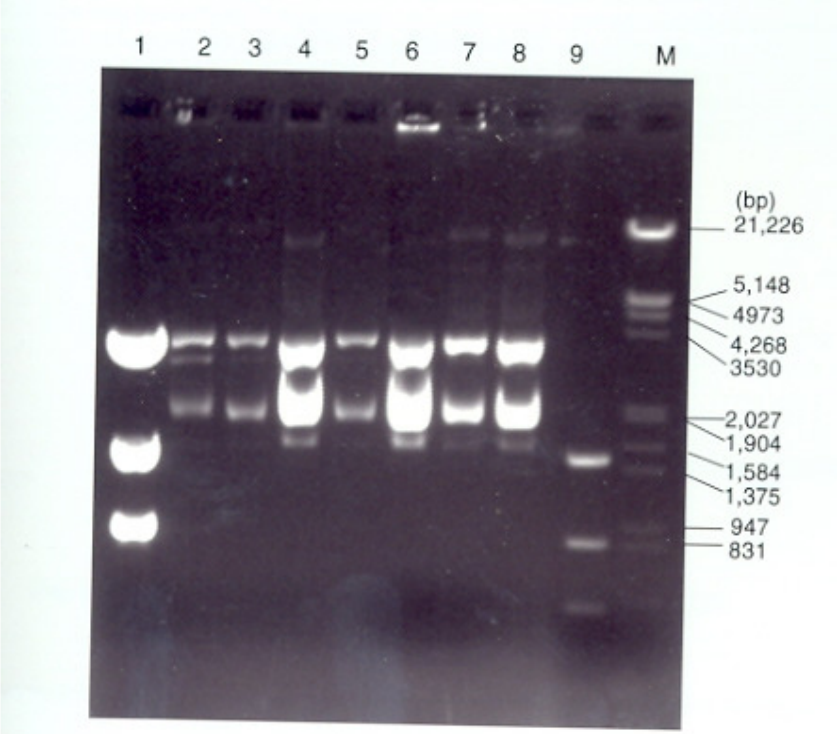
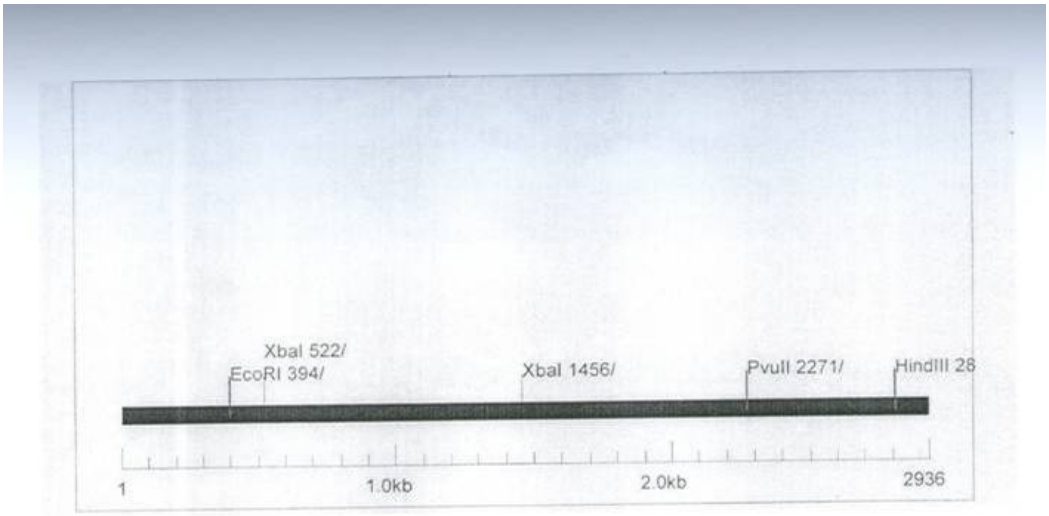


Plate 3b. Restriction analysis of pTZ57R/T clones with *Xba* I and *Bam* HI

Plate 3b. Restriction analysis of pTZ57R/T clones with Xba I and Bam HI

Fig. 2. Nucleotide sequence of the insert in clone pKM 225

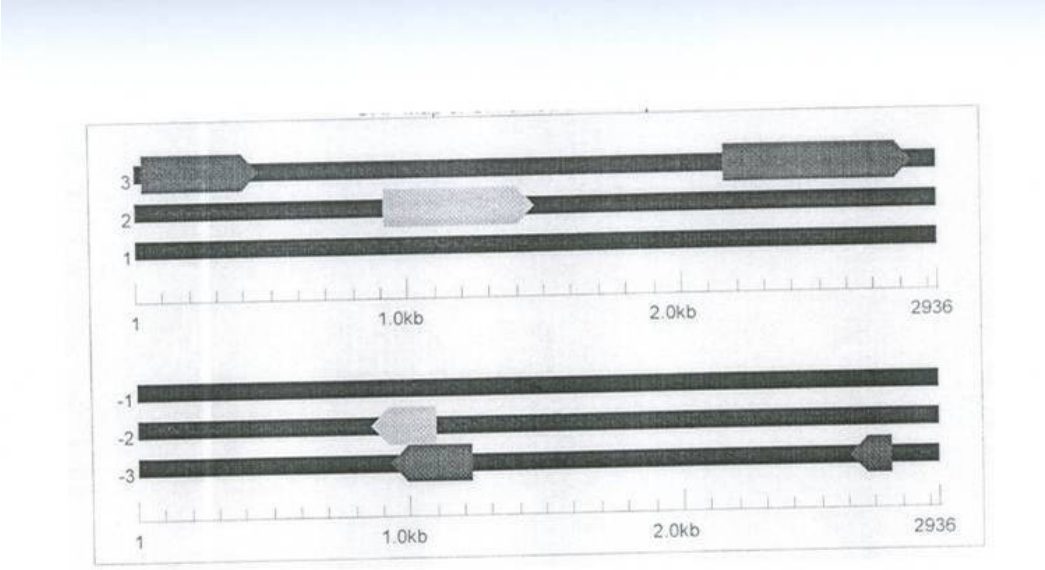
Fig.2 Nucleotide of the insert in clone pKM 225



Sequence Name : pKM225
Organism : Petunia

Fig.3. Restriction map of pKM225

Fig.3. Restriction map of KM225



Sequence Name : pKM225
Organism : Petunia

Fig.4. ORF Map of pKM225 DNA sequence

Fig.4. ORF Map of pKM225 DNA Sequence

Fig.5. Translation of pKM 225

Table 2. Sequences showing homology to the cloned insert

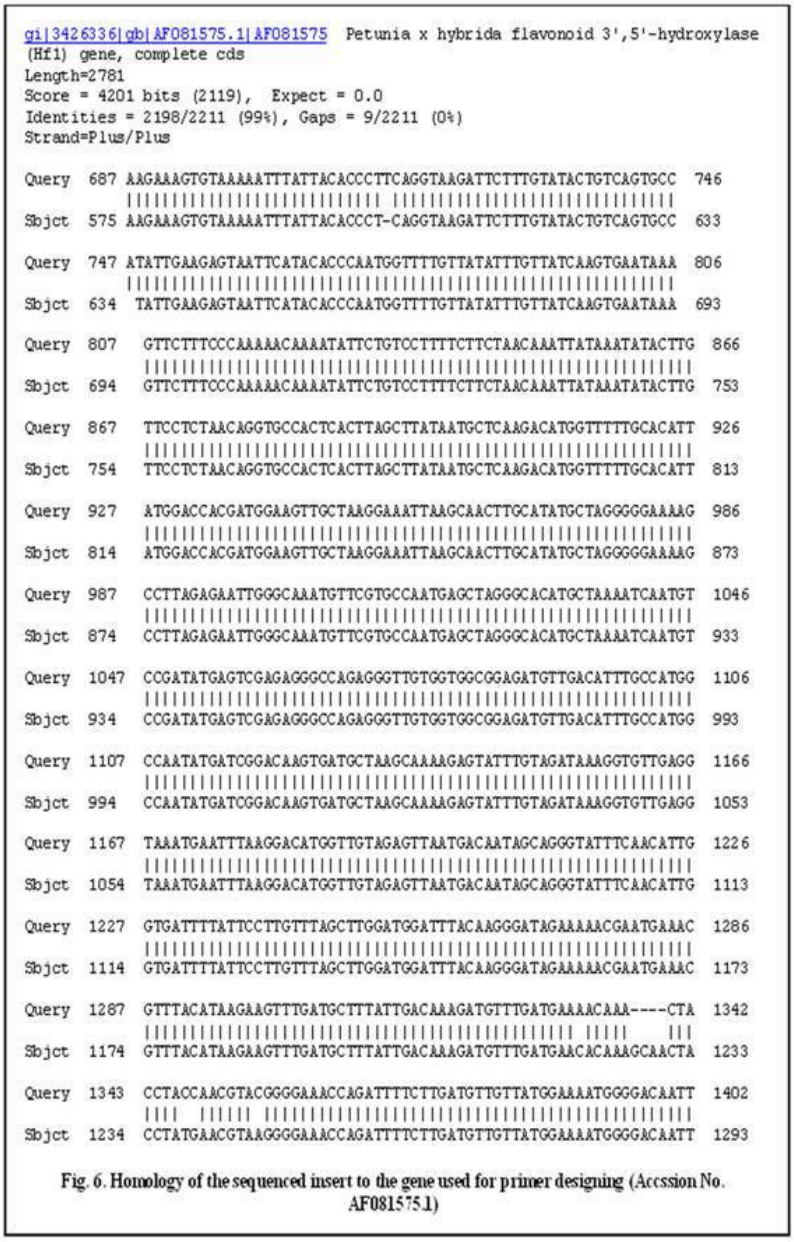
Accession No.	Gene	% homology	E value
AF081575.1	*Petunia x hybrida* flavonoid hydroxylase cds	99	0.0
222545.1	*P. hybrida* flavonoid 3'5'-hydrolase mRNA	99	0.0
D14588.1	*Petunia hybrida* F3'5'H mRNA for flavonoid 3'5' complete cds	99	0.0
AYZ455.1	*Petunia x hybrida* flavonoid 3'5'-hydroxylase mRNA cds	99	0.0
X71130.1	*P. hybrida* mRNA for P450 hydroxylase	98	0.0
Z22544.1	*P. hybrida* flavonoid 3'5'-hydroxylase mRNA	95	0.0
AB078514.1	*Nierembergia* sp. NB17f3'5'h mRNA for flavonoid complete cds	88	0.0
AF313490.1	*Lycianthes ratonnei* flavonoid 3'5'-hydrolase cds	86	2e-154

4.4 Conserved domains and blast results of the amino acid sequence

Conserved domain of the amino acid of the F3'5'H gene in pKM225 was found using www.ncbi.nih.nlm.gov showed that it belongs to a family pfam00067, P450 with 49.5 per cent homology. Cytochrome P450 which are involved in the oxidative degradation of various compounds. Structure is mostly alpha and binds a heme cofactor (Fig. 7). The conserved sequence is given in Fig. 8. The results of the amino acid sequence blast using the tblastx (NCBI) are given in Fig. 9. The amino acid blast result showed that the first 15 were showing higher homology to F3'5'H genes (Fig. 9). The amino acid sequence of the sequenced gene in the present study shows 90 per cent homology with p450 region of the petunia gene which was used to design the primers (Fig. 10).

4.5 Cloning of F3'5'H gene to plant expression vector

As the insert from pTZ57R/T could not be used, the PCR amplicon was eluted for cloning into plant expression vector. The presence of the internal *Xba* I sites had to be considered and therefore partial restriction was used to restrict PCR amplicon and after electrophoresis only the band corresponding to 2.9 kb size was eluted and purified for complete digestion with *Bam* HI. The ligated product with pHS-100 transferred to *E. coli* DH5 α was streaked on selection media of LB +Kan50 mg/lt. Clones, which showed growth, were used for confirmation through partial restriction with *Xba* I and *Bam* H I. Among the 9 clones restricted 3 showed restriction of 2.9 kb fragment (Plate 4a) which were further confirmed for the presence of insert by PCR amplification. PCR amplification in the three clones tested pKP 1220, 1222, 1225 showed 2.9 kb amplicon (Plate 4b). Restriction map of the construct pKP1225 containing full length F3'5'H gene in pHS100 is given in Fig. 11.



gi|3426336|gb|AF081575.1|AF081575 Petunia x hybrida flavonoid 3',5'-hydroxylase (Hf1) gene, complete cds

Length=2781
Score = 4201 bits (2119), Expect = 0.0
Identities = 2198/2211 (99%), Gaps = 9/2211 (0%)
Strand=Plus/Plus

```
Query  687 AAGAAAGTGTAAAAATTTATTACACCCTTCAGGTAAGATTCTTTGTATACTGTCAGTGCC  746
           ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  575 AAGAAAGTGTAAAAATTTATTACACCCT-CAGGTAAGATTCTTTGTATACTGTCAGTGCC  633

Query  747 ATATTGAAGAGTAATTCATACACCCAATGGTTTTGTTATATTTGTTATCAAGTGAATAAA  806
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  634  TATTGAAGAGTAATTCATACACCCAATGGTTTTGTTATATTTGTTATCAAGTGAATAAA  693

Query  807   GTTCTTTCCCAAAAACAAAATATTCTGTCCTTTTCTTCTAACAAATTATAAATATACTTG  866
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  694   GTTCTTTCCCAAAAACAAAATATTCTGTCCTTTTCTTCTAACAAATTATAAATATACTTG  753

Query  867   TTCCTCTAACAGGTGCCACTCACTTAGCTTATAATGCTCAAGACATGGTTTTTGCACATT  926
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  754   TTCCTCTAACAGGTGCCACTCACTTAGCTTATAATGCTCAAGACATGGTTTTTGCACATT  813

Query  927   ATGGACCACGATGGAAGTTGCTAAGGAAATTAAGCAACTTGCATATGCTAGGGGGAAAAG  986
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  814   ATGGACCACGATGGAAGTTGCTAAGGAAATTAAGCAACTTGCATATGCTAGGGGGAAAAG  873

Query  987   CCTTAGAGAATTGGGCAAATGTTCGTGCCAATGAGCTAGGGCACATGCTAAAATCAATGT  1046
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  874   CCTTAGAGAATTGGGCAAATGTTCGTGCCAATGAGCTAGGGCACATGCTAAAATCAATGT  933

Query  1047  CCGATATGAGTCGAGAGGGCCAGAGGGTTGTGGTGGCGGAGATGTTGACATTTGCCATGG  1106
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  934   CCGATATGAGTCGAGAGGGCCAGAGGGTTGTGGTGGCGGAGATGTTGACATTTGCCATGG  993

Query  1107  CCAATATGATCGGACAAGTGATGCTAAGCAAAAGAGTATTTGTAGATAAAGGTGTTGAGG  1166
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  994   CCAATATGATCGGACAAGTGATGCTAAGCAAAAGAGTATTTGTAGATAAAGGTGTTGAGG  1053

Query  1167  TAAATGAATTTAAGGACATGGTTGTAGAGTTAATGACAATAGCAGGGTATTTCAACATTG  1226
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1054  TAAATGAATTTAAGGACATGGTTGTAGAGTTAATGACAATAGCAGGGTATTTCAACATTG  1113

Query  1227  GTGATTTTATTCCTTGTTTAGCTTGGATGGATTTACAAGGGATAGAAAAACGAATGAAAC  1286
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1114  GTGATTTTATTCCTTGTTTAGCTTGGATGGATTTACAAGGGATAGAAAAACGAATGAAAC  1173

Query  1287  GTTTACATAAGAAGTTTGATGCTTTATTGACAAAGATGTTTGATGAAAACAAA----CTA  1342
              |||||||||||||||||||||||||||||||||||||||||||||||| |||||    |||
Sbjct  1174  GTTTACATAAGAAGTTTGATGCTTTATTGACAAAGATGTTTGATGAACACAAAGCAACTA  1233

Query  1343  CCTACCAACGTACGGGGAAACCAGATTTTCTTGATGTTGTTATGGAAAATGGGGACAATT  1402
              ||||  |||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1234  CCTATGAACGTAAGGGGAAACCAGATTTTCTTGATGTTGTTATGGAAAATGGGGACAATT  1293
```

Fig. 6. Homology of the sequenced insert to the gene used for primer designing (Accssion No. AF081575.1)

Fig.6. (Contd..).. Homology of the sequenced insert to the gene used for primer designing (accession NO. AF081575.1)

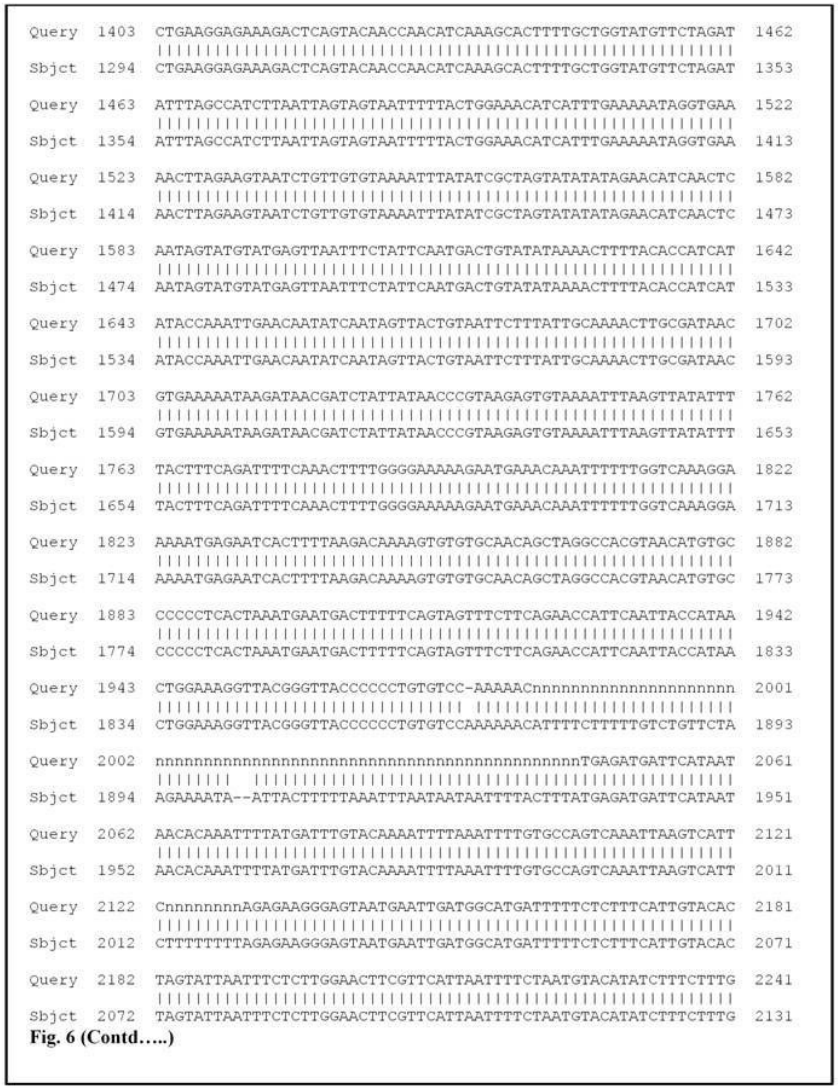


Fig. 6 (Contd…..)

Fig.6. (Contd..)

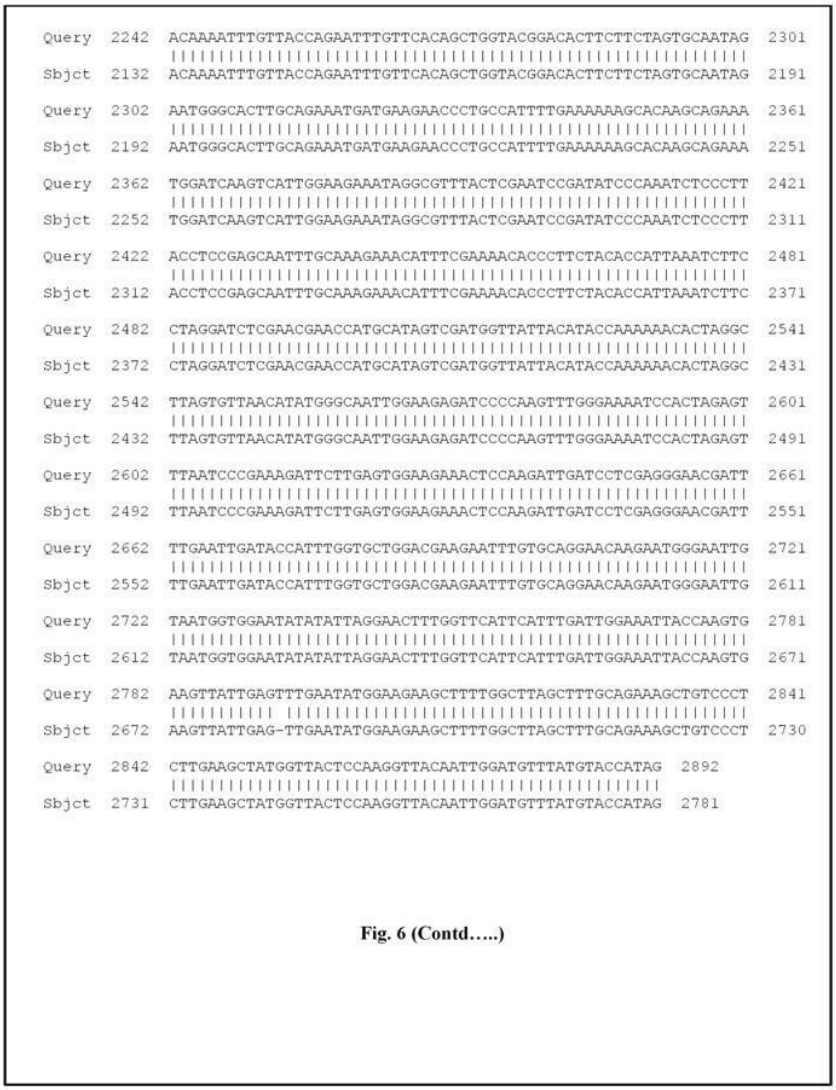
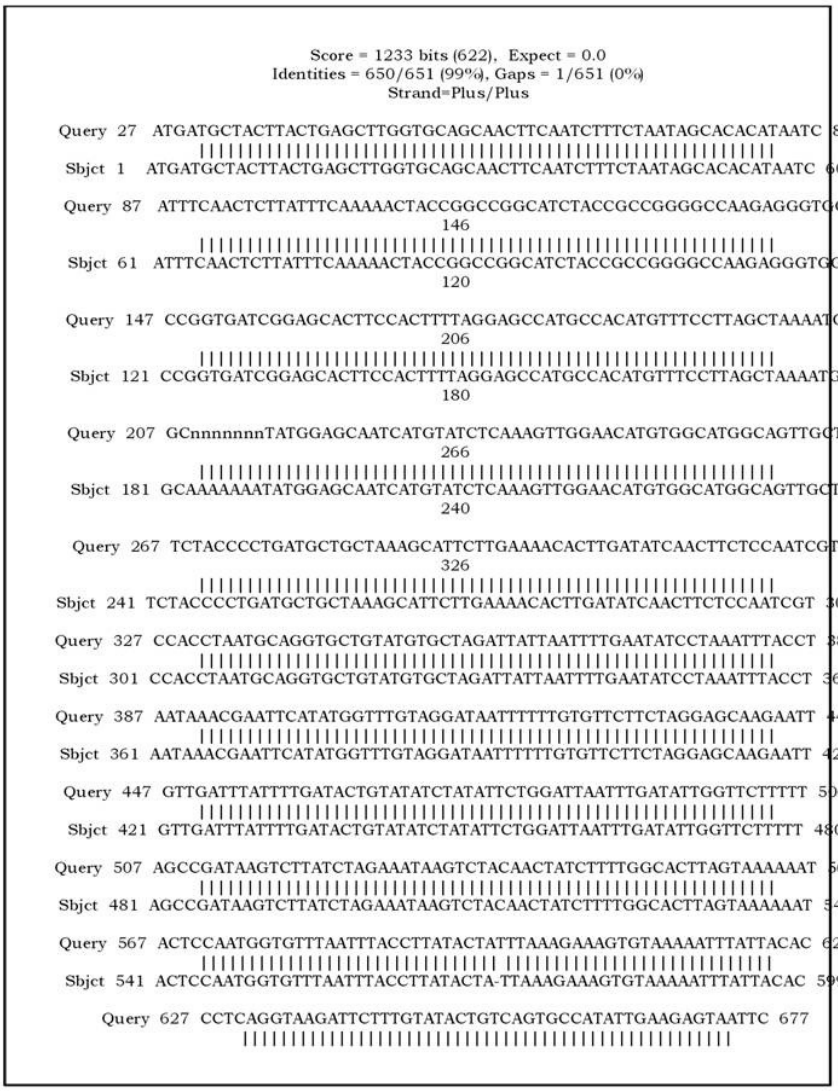


Fig. 6 (Contd…..)

Fig.6. (Contd..)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Identities = 650/651 (99%), Gaps = 1/651 (0%)
Strand=Plus/Plus
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Sbjct 1 ATGATGCTACTTACTGAGCTTGGTGCAGCAACTTCAATCTTTCTAATAGCACACATAATC 60
Query 87 ATTTCAACTCTTATTTCAAAAACTACCGGCCGGCATCTACCGCCGGGGCCAAGAGGGTGG 146
Sbjct 61 ATTTCAACTCTTATTTCAAAAACTACCGGCCGGCATCTACCGCCGGGGCCAAGAGGGTGG 120
Query 147 CCGGTGATCGGAGCACTTCCACTTTTAGGAGCCATGCCACATGTTTCCTTAGCTAAAATG 206
Sbjct 121 CCGGTGATCGGAGCACTTCCACTTTTAGGAGCCATGCCACATGTTTCCTTAGCTAAAATG 180
Query 207 GCnnnnnnnTATGGAGCAATCATGTATCTCAAAGTTGGAACATGTGGCATGGCAGTTGCT 266
Sbjct 181 GCAAAAAAATATGGAGCAATCATGTATCTCAAAGTTGGAACATGTGGCATGGCAGTTGCT 240
Query 267 TCTACCCCTGATGCTGCTAAAGCATTCTTGAAAACACTTGATATCAACTTCTCCAATCGT 326
Sbjct 241 TCTACCCCTGATGCTGCTAAAGCATTCTTGAAAACACTTGATATCAACTTCTCCAATCGT 300
Query 327 CCACCTAATGCAGGTGCTGTATGTGCTAGATTATTAATTTTGAATATCCTAAATTTACCT 386
Sbjct 301 CCACCTAATGCAGGTGCTGTATGTGCTAGATTATTAATTTTGAATATCCTAAATTTACCT 360
Query 387 AATAAACGAATTCATATGGTTTGTAGGATAATTTTTTGTGTTCTTCTAGGAGCAAGAATT 446
Sbjct 361 AATAAACGAATTCATATGGTTTGTAGGATAATTTTTTGTGTTCTTCTAGGAGCAAGAATT 420
Query 447 GTTGATTTATTTTGATACTGTATATCTATATTCTGGATTAATTTGATATTGGTTCTTTTT 506
Sbjct 421 GTTGATTTATTTTGATACTGTATATCTATATTCTGGATTAATTTGATATTGGTTCTTTTT 480
Query 507 AGCCGATAAGTCTTATCTAGAAATAAGTCTACAACTATCTTTTGGCACTTAGTAAAAAAT 566
Sbjct 481 AGCCGATAAGTCTTATCTAGAAATAAGTCTACAACTATCTTTTGGCACTTAGTAAAAAAT 540
Query 567 ACTCCAATGGTGTTTAATTTACCTTATACTATTTAAAGAAAGTGTAAAAATTTATTACAC 626
Sbjct 541 ACTCCAATGGTGTTTAATTTACCTTATACTA-TTAAAGAAAGTGTAAAAATTTATTACAC 599
Query 627 CCTCAGGTAAGATTCTTTGTATACTGTCAGTGCCATATTGAAGAGTAATTC 677

Fig.6

PSSMs producing significant alignments:	Score (bits)	E value
gnl|CDD|25378 pfam00067, p450, Cytochrome P450.	105	1e-23

CD-Length = 461 residues, 49.5% aligned

Score = 105 bits (263), Expect = 1e-23

```
Query: 34  PPGPRGWPVIGALPLLG--AMPHVSLAKMAKKYGAIMYLKVGTCATHLAYNAQDM----- 86
Sbjct: 1   PPGPTPLPLFGNLLQLGRKGNLHSVLTKLQKKYGPIFRLYLGSRPVVVLSGPEAVKEVLI 60

Query: 87  ----------------------VFAHYGPRWKLLRKLSN--LHMLGGKALENWANV 118
Sbjct: 61  KEGEEFSGRPDEPWFYTLLGPFLGKGIAFANGPRWRQLRRFLTPTFRSFGKLSLEPIVEE 120

Query: 119 RANELGHMLKSMSDMSREGQRVVVAEMLTFAMANMIGQVMLSKRVFVDKGVEVNEFKDMV 178
Sbjct: 121 EARDLVEKLRKTAG---ESGVIDITDLLSKAALNVICSILFGKRFDSLEDPEFLELVKAV 177

Query: 179 VELMTIAGYF--NIGDFIPCLAWMDLQGIEKRMKRLHKKFDALLTKMFDENK 228
Sbjct: 178 QELSSLLSSPSPQLLDLFPLLKYF-PGPHLRKFKRARKKIKDLLDKLIEERR 228
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Fig. 7. Conserved Domain

Fig.7: Conserved Domain

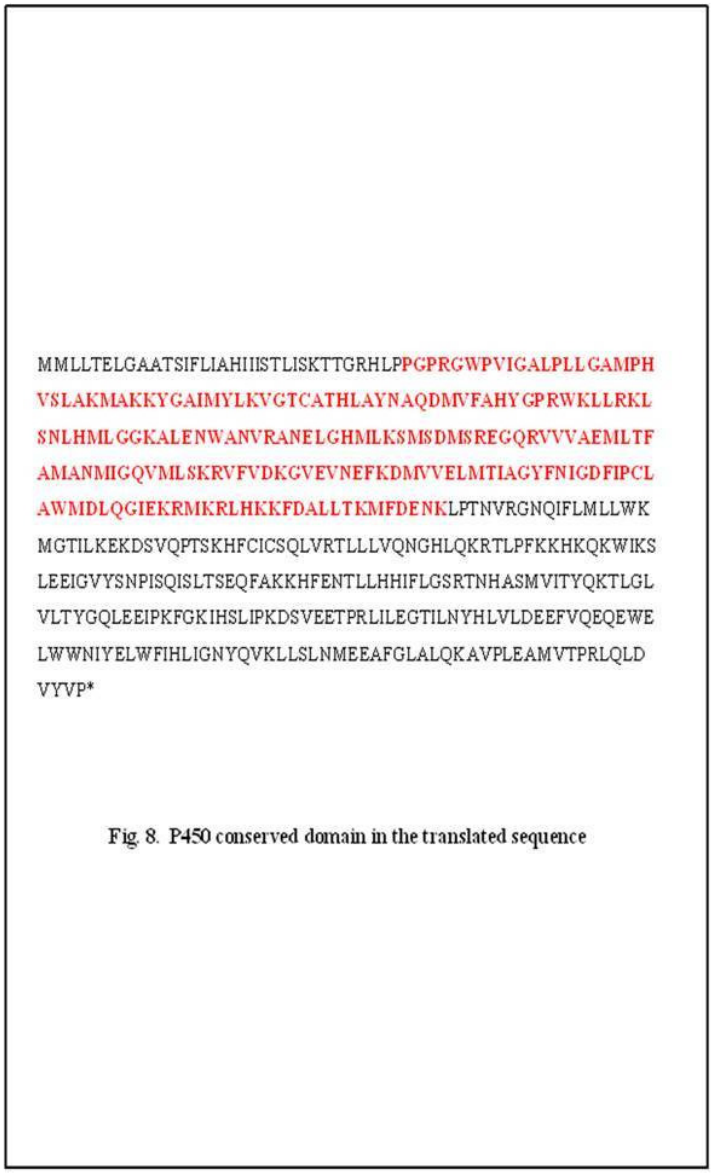
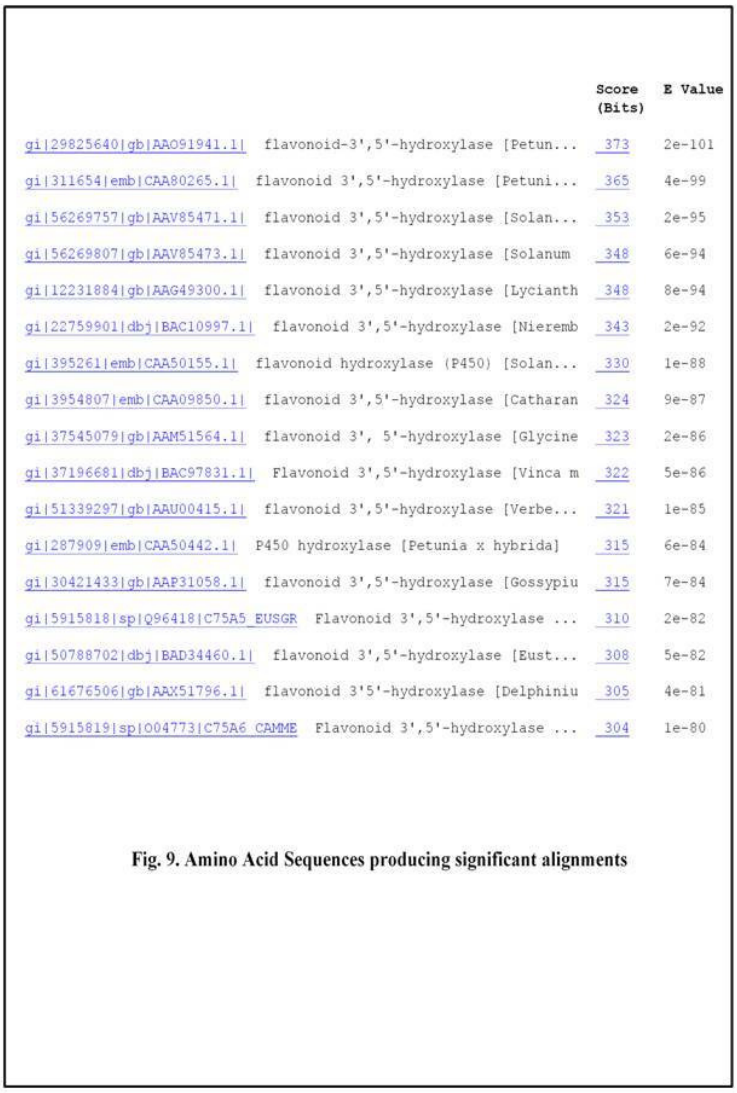


Fig. 8. P450 conserved domain in the translated sequence

Fig.8. P450 conserved domain in the translated sequence



	Score (Bits)	E Value
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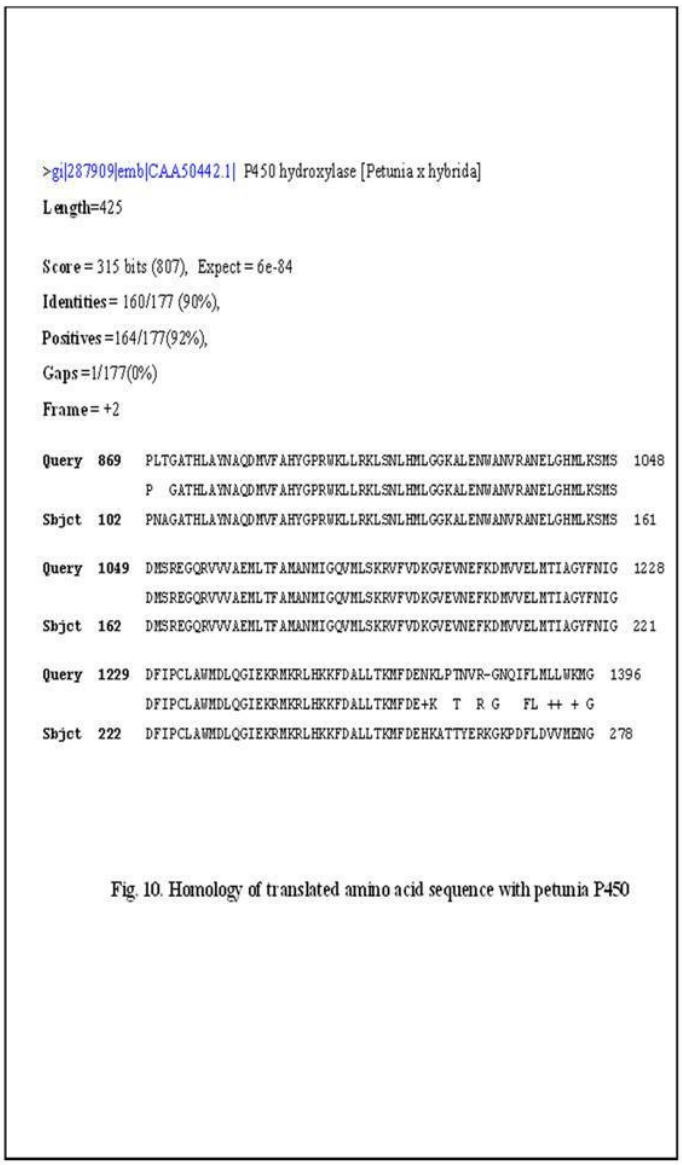
gi|311654|emb|CAA80265.1| flavonoid 3',5'-hydroxylase [Petuni...	365	4e-99
gi|56269757|gb|AAV85471.1| flavonoid 3',5'-hydroxylase [Solan...	353	2e-95
gi|56269807|gb|AAV85473.1| flavonoid 3',5'-hydroxylase [Solanum	348	6e-94
gi|12231884|gb|AAG49300.1| flavonoid 3',5'-hydroxylase [Lycianth	348	8e-94
gi|22759901|dbj|BAC10997.1| flavonoid 3',5'-hydroxylase [Nieremb	343	2e-92
gi|395261|emb|CAA50155.1| flavonoid hydroxylase (P450) [Solan...	330	1e-88
gi|3954807|emb|CAA09850.1| flavonoid 3',5'-hydroxylase [Catharan	324	9e-87
gi|37545079|gb|AAM51564.1| flavonoid 3', 5'-hydroxylase [Glycine	323	2e-86
gi|37196681|dbj|BAC97831.1| Flavonoid 3',5'-hydroxylase [Vinca m	322	5e-86
gi|51339297|gb|AAU00415.1| flavonoid 3',5'-hydroxylase [Verbe...	321	1e-85
gi|287909|emb|CAA50442.1| P450 hydroxylase [Petunia x hybrida]	315	6e-84
gi|30421433|gb|AAP31058.1| flavonoid 3',5'-hydroxylase [Gossypiu	315	7e-84
gi|5915818|sp|Q96418|C75A5_EUSGR Flavonoid 3',5'-hydroxylase ...	310	2e-82
gi|50788702|dbj|BAD34460.1| flavonoid 3',5'-hydroxylase [Eust...	308	5e-82
gi|61676506|gb|AAX51796.1| flavonoid 3'5'-hydroxylase [Delphiniu	305	4e-81
gi|5915819|sp|O04773|C75A6_CAMME Flavonoid 3',5'-hydroxylase ...	304	1e-80

Fig. 9. Amino Acid Sequences producing significant alignments

Fig.9. Amino Acid Sequences producing significant alignments



>gi|287909|emb|CAA50442.1| P450 hydroxylase [Petunia x hybrida]

Length=425

Score = 315 bits (807), Expect = 6e-84

Identities = 160/177 (90%),

Positives = 164/177(92%),

Gaps = 1/177(0%)

Frame = +2

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Sbjct  102   PNAGATHLAYNAQDMVFAHYGPRWKLLRKLSNLHMLGGKALENWANVRANELGHMLKSMS  161

Query  1049  DMSREGQRVVVAEMLTFAMANMIGQVMLSKRVFVDKGVEVNEFKDMVVELMTIAGYFNIG  1228
             DMSREGQRVVVAEMLTFAMANMIGQVMLSKRVFVDKGVEVNEFKDMVVELMTIAGYFNIG
Sbjct  162   DMSREGQRVVVAEMLTFAMANMIGQVMLSKRVFVDKGVEVNEFKDMVVELMTIAGYFNIG  221

Query  1229  DFIPCLAWMDLQGIEKRMKRLHKKFDALLTKMFDENKLPTNVR-GNQIFLMLLWKMG  1396
             DFIPCLAWMDLQGIEKRMKRLHKKFDALLTKMFDE+K  T  R G   FL ++ + G
Sbjct  222   DFIPCLAWMDLQGIEKRMKRLHKKFDALLTKMFDEHKATTYERKGKPDFLDVVMENG  278
```

Fig. 10. Homology of translated amino acid sequence with petunia P450

Fig.10. Homology of translated amino acid sequence with petunia P450

4.6 *Agrobacterium* transformation through tri parental mating

The triparental mating with the donor, *E. coli* DH5α with cloned vector, recepient, *A. tumafaciens*, AH 4404, and helper *pRK*2013 was done and the proportion of Donor : Recipient : Helper (D:R:H) used for patch mating was 1:1:1 and 2:1:1. It was observed that in the selection media for all the three (D, R, H) *i.e.* kanamycin (50 mg/lt) for *pHS*-100 vector and helper, streptomycin (100 mg/lt) and rifamycin (25 mg/lt) for *AH*4404, there was growth in both the proportions mixed while all the three donor, helper and recipient individually streaked did not show growth.

4.7 *In vitro* transformation and *in planta* transformation

4.7.1 *In vitro* transformation

In vitro transformation of the four selected cotton genotypes, two brown pigmented and two white were transformed following the method described earlier (Plate 5a). The number of explants/meristematic shoot co-cultivated and the number of seedlings established in each genotype is presented in Table 3.

The To plants were observed for morphological changes in pigment colour for the expression of F3'5'H gene. All the seedlings are green without the pigment colour changes. The flowers have been selfed to screen the T_1 generations for transformation morphologically.

4.7.2 *In planta* transformation

The seedlings in pots were co-cultivated with solid *Agrobacterium* culture after cutting the meristematic tip with sharpe knife (Plate 5b). The number of seedlings co-cultivated, number of seedlings established and the number of seedlings showing pigment colour changes are presented in Table 4. Among the 4 genotypes used for *in planta* co-cultivation, in the white linted genotype PA-255, 4 plants out of 112 plants established had colour changes in the leaf, veins, squares bolls and stem and had purple colour compared to the green colour in the control (Plate 6a, 6b and 6d). The flower of one of the transgenic plant did not have the petal spot invariably present in the *desi* cotton genotypes (Plate 6c). Two of the MB-25 brown linted genotypes showed lint colour variation. One T_0 plant of MB-25 which had originally brown lint after transformation showed pigment change towards sea blue shade (Plate 7a). One more plant showed lint colour of pink (Plate 7a). These plants were however similar to parent *i.e.* green is all other tissues like leaves and stem.

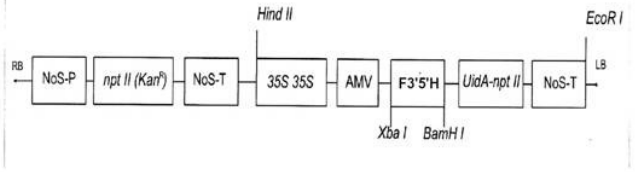


Fig. 11. Restriction map of construct pKP1225 containing full length F3'5'H gene in pHS100

Fig.11. Restriction map of construct pKP1225 containing full length F3’5’H gene in pHS100

LEGEND

Plate 4a. Restriction analysis of pHS-100 clones with *Xba* I and *Bam* HI

M : λ DNA/*Eco* RI + Hind III double digest

1 to 9 : Restriction of pHS-100 clones with *Xba* I and *Bam* H I

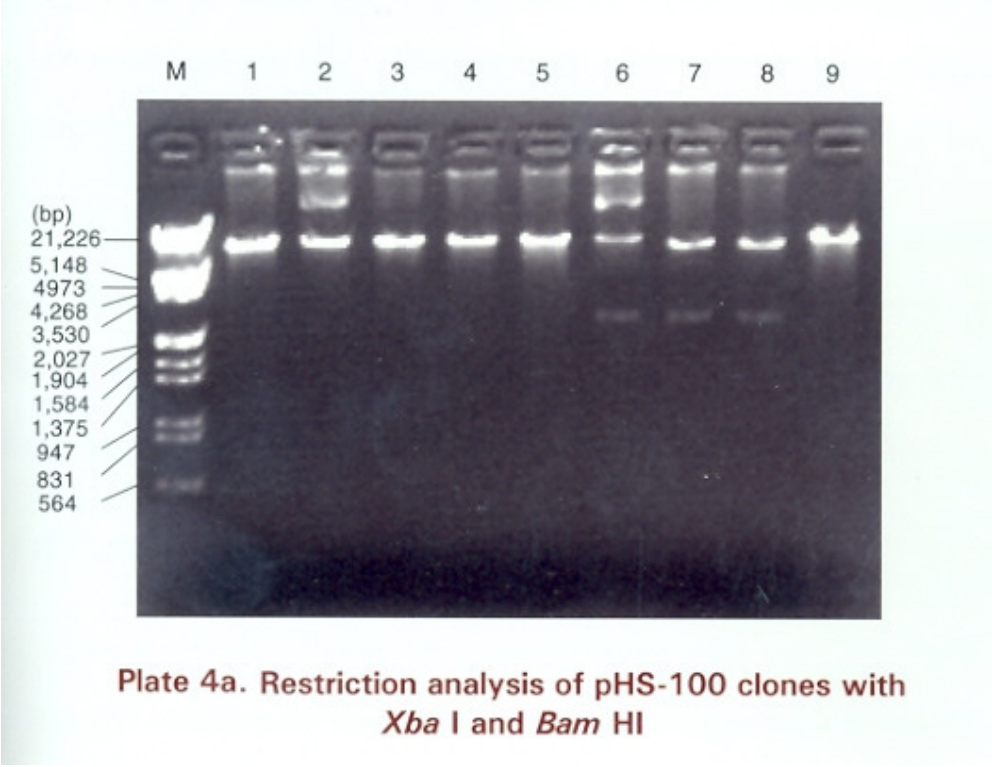


Plate 4a. Restriction analysis of pHS-100 clones withXba I and Bam HI

Legend
Plate 4b. PCR analysis of pHS 100 clones

M	: λ DNA/*Eco* RI + Hind III double digest
1	: PCR amplification of pKP 1220
2	: PCR amplification of pKP 1222
3	: PCR amplification of pKP 1225

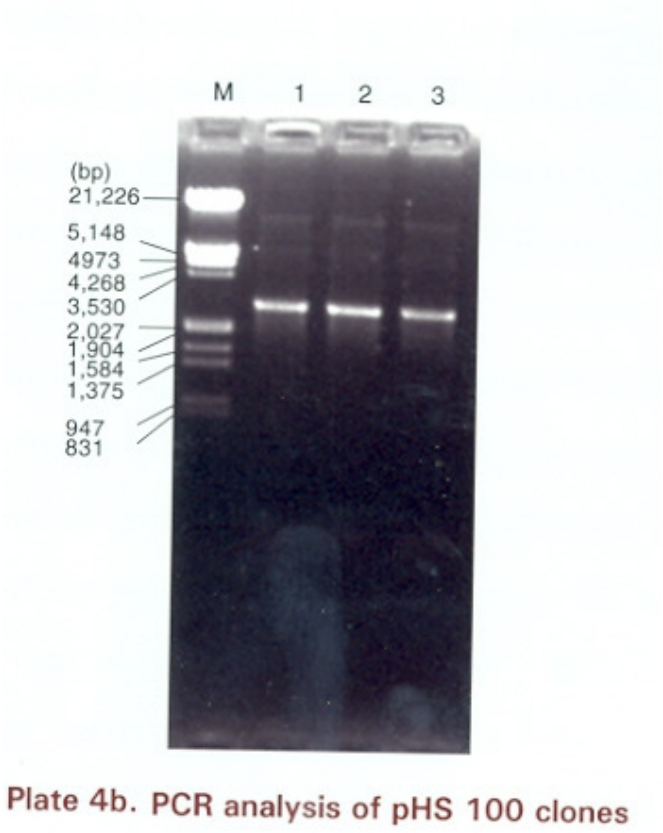


Plate 4b. PCR analysis of pHS 100 clones

Plate 4b. PCR analysis of pHS 100 clones

Plate 5a. *In vitro* transformation cotton seedlings

Plate 5a. *In Vitro* transformation cotton seedling

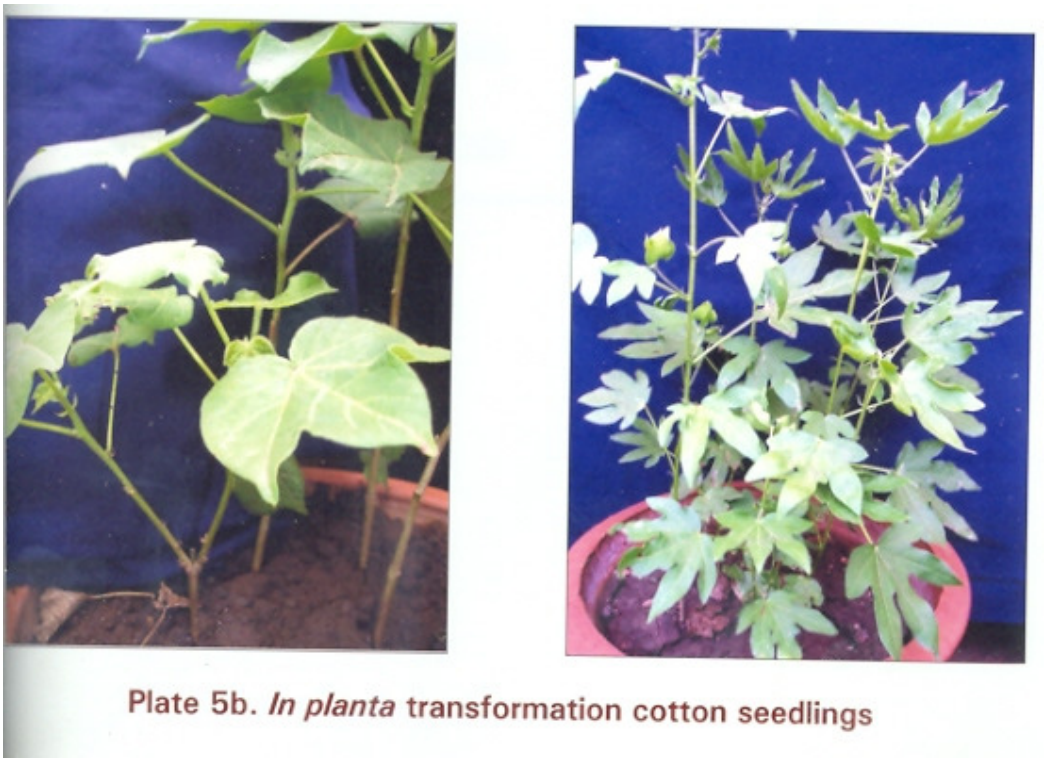


Plate 5b. *In planta* transformation cotton seedlings

Plate 5b. *In planta* transformation cotton seedlings

Table 3. Number of cotton explants co-cultivated, established and transformed *in vitro*

Genotype	Lint colour	Number of explants co-cultivated	Number of plants established	Number of plants transformed
Sahana	White	2296	882	Nil
MB-25	Brown	3560	478	Nil
PA-255	White	4340	584	Nil
DDCC-1	Almond	3250	542	Nil

Table 4. Number of cotton seedlings co-cultivated, established and transformed *in planta*

Genotype	Lint colour	Number of seedlings co-cultivated	Number of plants established	Number of plants transformed
Sahana	White	200	85	Nil
MB-25	Brown	200	117	2
PA-255	White	200	112	4
DDCC-1	Almond	200	95	Nil

LEGEND

Plate 6b. Bracts and flower bud

1. Transgenic
2. Control

Plate 6c. Flower

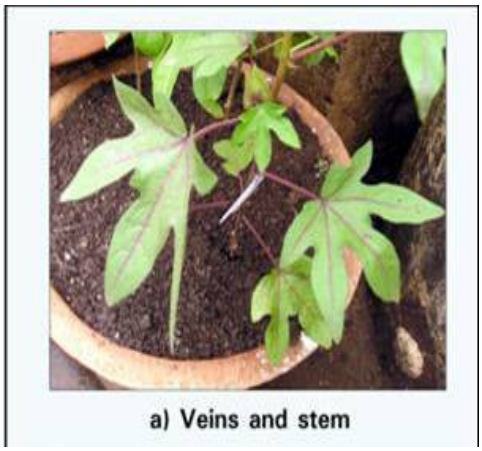
1. Transgenic
2. Control

Plate 6d. Bolls

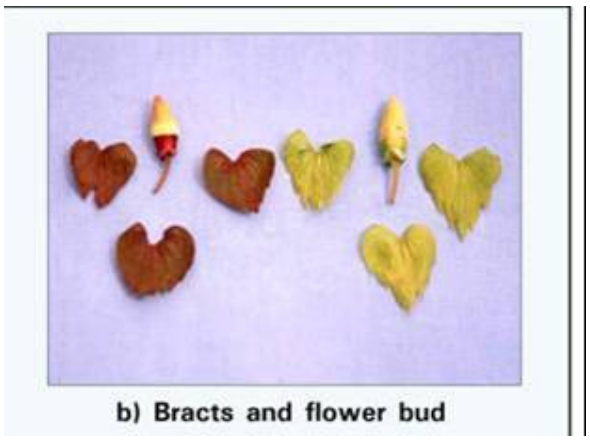
1. Control
2. Transgenic

Plate 6e.

1 to 4 : PCR amplification of 4 trasngenic *G. arboreum* plants

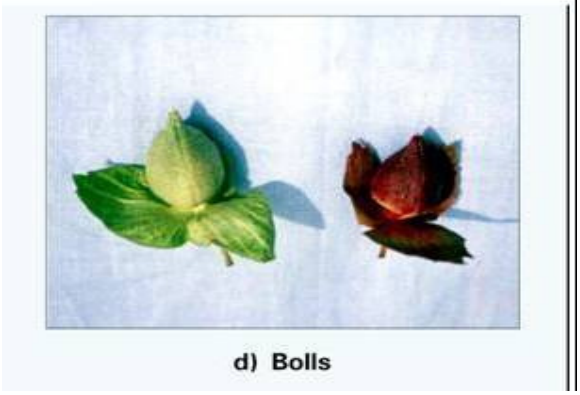


a) Veins and stem of ttransgenic plant



b)Bracts and flower bud

C) Flower



d)bolls

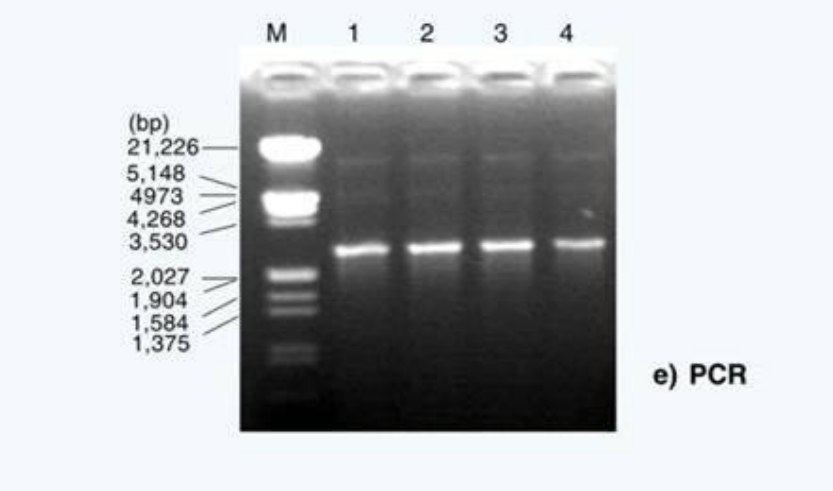


Plate 6. Pigment bchangesand PCR analysis in transgenic *G.arboreum* cotton genotype

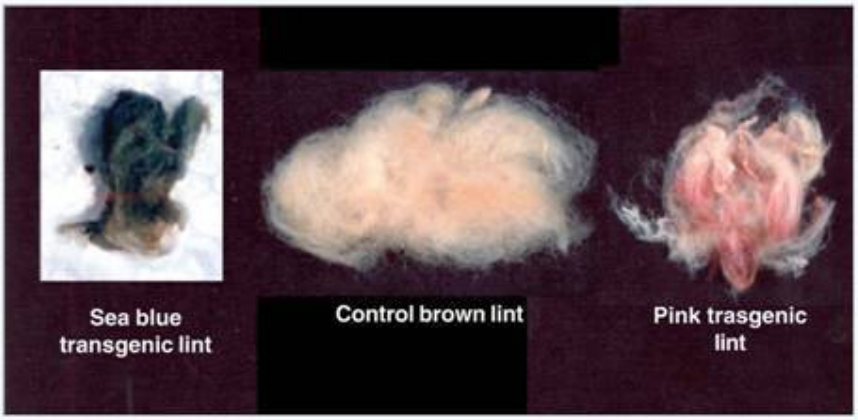


Plate 7a. Lint colour variation of transgenic *g.hirsutum* cotton genotype

LEGEND

Plate 7b. PCR analysis of transgenic *G. hirsutum* genotype

1. Transgenic sea blue linted plant
2. Control with brown lint
3. Transgenic pink linted plant

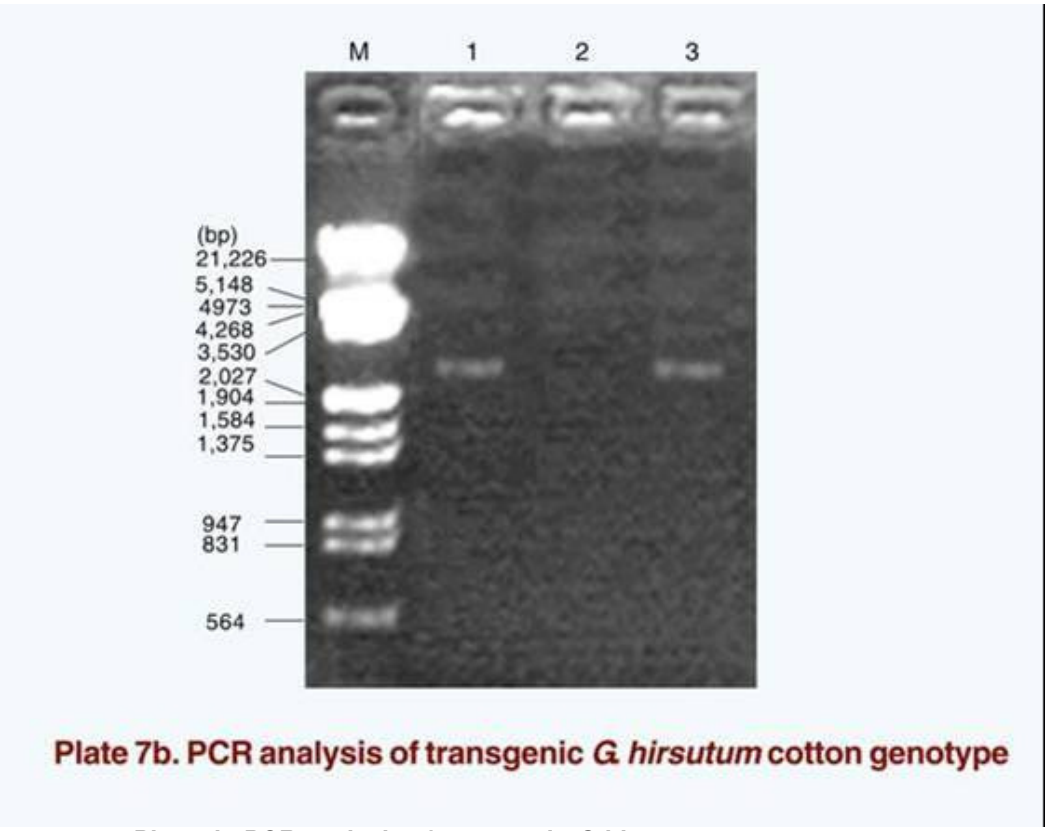


Plate 7b. PCR analysis of transgenic *G.hirsutum* cotton genotype

4.7.3 Confirmation of transformants

The leaf of the PA-255 with pigment colour change were used for DNA extraction and amplified for *npt* II primer. There was amplification in all four plants with purple coloured veins while there was no amplification in negative control. The same four plants also showed amplification for the complete 2.9 kb gene when specific primers were used for PCR amplification (Plate 6e). Amplification of the genomic DNA of the two plants with lint colour variation also showed the amplification of 2.9 kb gene with specific primers (Plate 7b).

V. DISCUSSION

Enormous amounts of cotton fibres are harvested every year to supply the market with textiles. A large part of this is dyed in different colours, which creates an environmental problem by the affluents of these dyes and an unnecessary expense. To avoid this, natural pigments can be expressed in the fibres and thereby make the fibres coloured. After decades of breeding only for natural colours in the fibres, brown, bronze brown and two varieties of green are commercially grown by Natural Cotton Inc., USA and few other countries including India. More of the most common colours, dyeing is still the only option which needs to be changed (Taylor, 2002). The potential of organically grown coloured cottons to meet the new market demand for such cottons to enhance aesthetic value, production of fashion textiles, double/triple value addition from use of organic coloured lint spun yarn, economic consideration in agriculture and premium price in trade and export, saving in dyeing cost, promotion of handlooms and artisan fabrics, medical and spiritual (in Peru) uses of coloured cottons, special purpose novelty niche cottons, have all given rise to the new inpetus. Sally Fox working for Natural Cottons Colours USA formed in 1989 started detailed basic studies in 1982 and developed several coloured linted genotypes for commercial sale and cultivation in specific location sin USA (Fox, 1987; Anonymous, 1992).

Three types of natural pigments, flavonoids, carotenoids and betalanios can provide a plant with the colours of the flower and other plant parts. By expressing some of them in the fibres of the cotton boll, a whole new range of colours can be introduced to the market. Anthocyanin is a sub-group of flavonoids. It's and aromatic molecular that is derived from the precursors Δ-coumatoyl-CoA (via the general phenylphopanoid pathway) and malonyl-coenzyme A (via the fatty acid pathway) (Winkel-Shirley, 2001). There are several derivatives within the anthocyanin family that give different colours (orange, red, crimson, scarlet, pink, violet and blue) depending on the number of their hydroxyl groups of the A and in partiuclar, the B-ring and by the degree of methylation of these hydroxyl groups (Forkmann, 1991). The biosynthetic pathway of anthocyanins is complex with several enzymes involved : Chalcone synthase (CHS), chalcone isemerase (CHI), flavanone 3-hydroxylase (FH3), flavonoid 3-hydroxylase (F3'H), flavonoid 3', 5'-hydrolyase (F3'5'H), dihydro flavonoid 4-reductase (DFR), anthocyanidin (ANS), 3-0-glycosyl transferase (3 GT) (Taylor, 2002).

CHS catalyses the condensation of the precursors to tetrahydroxychalone (yellow) which is transformed to a colourless naringenin by CHI. F3H is then converting the substance to dihydro-karmpeternol (DHK) which is hydroxylated to dihydroquerectin (DHQ) by F3'H. F3'5'H hydrolyation DHK to dihydromyricetin (DHM) or convert DHQ to DHM. The three dihydroflavonols are then reduced by DFR to flavan-3, 4-cisdiols (leucoantocyanidins). Oxidation, dehydration and glycosylation produce different coloured pigments for example red cyanidis-3-glucoside catalysed by ANS and 3GT (Holton and Cornish, 1995). The three-dimensional structure for CHS and CHI have been determined which provide an understanding of multiple decarboxylation, condensation reactions and intra molecular cyclization performed by these enzymes (Ferrer *et al*., 1999 and Jez *et al*., 1999).

From the first description of acid and base effects on plant pigments by Robert Boyle in 1664 to the characterization of structural and regulatory genes in the late 20th century, a wealth of information has been collected on the structure, chemical activity and biosynthesis of these compounds. In the recent years, much effort has been directed at elucidating the flavonoid biosynthetic pathway from a molecular genetic point of view. Many of the structural and regulatory genes are characterized and cloned from maize, shapdragon and petunia (Holton and Cornish, 1995). These cloned genes have been used for transformation and flavonoid pathway modulation is plant tissues by expression of novel sense derivatives of genes not present in the gene pool of the target plant to open the pathway to new metabolites; expression of sense derivatives of a suitable gene to open the pathway to new metabolites by overcoming genetic blocks or rate limiting steps and gene silencing to down regulate synthesis of undesired flavonoids.

Because of the commercial value of flowers, their pigmentation has also been a subject of applied research since four centuries, with respect to pigment modification to get varied coloured flowers not available in the respective species. The recombinant DNA technology and biotechnological developments can be superimposed in pigment modification

in cotton fiber to develop coloured cotton with new shade/colour. This would help in the commercial viability of coloured cottons. This would be possible if the genes for anthocyanin biosynthesis already exist in the genome. Anthocyanin is a common pigment present in many species and has been shown to exist in cotton (Singh and Singh, 1982).

5.1 Isolation and cloning of flavonoids 3'5' hydroxylase gene from petunia

Efforts to engineer flower colour have led to some interesting developments in the last few years. The hydroxylation pattern of the B ring of anthocyanins is a major determinant of the colour of these pigments. All flavonoids carry a hydroxyl group at the A position, including the pink-to-red cyanidins based pigments. Hydroxylation at two variable positions is controlled by the P450 enzymes; F3'H, which leads to brick-red to orange pelargonidins, and F3'5'H, required for synthesis of purple and blue delphinidins. Two F3'5'H genes from petunia were isolated based on the sequence homology to the other P450s, a pattern of high-level expression in flowers and correlation with the hf_1 and hf_2 loci by Holton *et al*. in 1993. Cloning of the first F3'H gene took a bit longer, but a petunia gene was eventually isolated using a similar approach (Brugliera *et al*., 1999). Later the F3'H gene in *Arabidopsis* also was identified by chromosome walking to the *tt*7 locus.

In the present study primers were designed using the available sequence of the gene encoding Flavonoid 3'5' Hydroxylase to amplify the gene from purple coloured native petunia genotype. The specific amplicon of 2.9 kb was cloned in pTZ57R/T and sequenced. The insert from this cloning vector could not be used for further cloning as both the size of insert and vector was almost same. Therefore the primers having the restriction sites *Xba* I and *Bam* HI in forward and reverse primers respectively, were used for PCR amplification. The restricted products of both the amplicon and vector, pHS-100 were subjected to ligation. As the sequence cloned in this study for F3'5'H gene had two internal *Xba* I restriction sites at 5226 bp and 1456 bp, PCR amplicon was subjected to partial restriction with *Xba* I and only the band corresponding to 2.9 kb was eluted for further cloning by which successful positive clones were isoalted. In many other studies reported different approaches such as transposon tagging and position cloning have been used to isolate addition mutations in gene either directly or indirectly involved in flavonoid biosynthesis (Wisman *et al*., 1998; Kubo *et al*., 1999; Borevitz *et al*., 2000).

5.2 Sequencing and protein data

The sequencing of the pTZ57R/T clone for the cloned fragment using M13 primer was done by prime walking. The sequence blast results using NCBI showed that there was 99 per cent homology to the previously deposited petunia F3'5'H genes (Table 2). This confirmed that in the present study also the cloned amplicon is F3'5'H gene and is complete as it has all the introns and exons in the sequenced fragment (Fig. 2) and codes for 475 amino acids. The conserved amino acid domain between 34 to 228 is homologous to the P450 gene conserved domain with 49.5 per cent homology. The amino acid of the present study shows 90 per cent homology to the conserved amino acid P450 region reconfirming the cloned gene of the present study to be F3'5'H. Amino acid blast results also indicates the same. Cytochrome P450 (Cyt P450) dependent monooxygenase are a large group of membrane-bound heme-containing enzyme that are involved in a range of NADPH- and O_2-dependent hydroxylation reactions. Plant Cyt P450s have proved more difficult to purify. Only a limited number of plant Cyt P450 genes could be successfully purified and reconstituted (Mituzutani *et al*., 1993; Jeltsch, 1993; Koch *et al*., 1995). Therefore, once the sequence is known isolation of the gene is possible through PCR amplification using specific primers.

The F3'5'H gene isolated in the present study was transferred to *A. tumafaciens*, LBA 4404 by triparental mating using the helper *pRK*2013..

5.3 Transformation and expression of f3'5'h gene in cotton

Genetic modification through genetic engineering has been restricted to a few genotypes of cotton as *in vitro* regeneration through somatic cells is highly genotype specific. Most of the presently developed cotton transgenics for bollworm resistance and herbicide resistance were done using Coker 312, an American land race in which somatic regeneration has been reported. Subsequently, this genotype has been used as donor parent in backcross breeding to transfer the desired traits to other genotypes which takes at least 3-4 years and is associated with the disadvantage of undesirable linkage drag.

Earlier studies in our Institute, Indian cotton genotypes were tried for regeneration through somatic cells *in vitro*, but none of them has shown regeneration (Sureshkumar *et al*., 2002). Alternate protocols have been developed for the production of shoots and intact plants from pre-existing shoot meristem of isolated seedling plumules (Gould *et al*.,1991; Gould and Smith,1988; Katageri *et al*.,1998; Veluthambi *et al*.,1989). There are also many successful reports on meristem based methods using *Agrobacterium* mediated transformation of pea (Hussay *et al*., 1989), sunflower (Bidney *et al*., 1992), corn (Gould *et al*., 1991), banana (May *et al*., 1995), tobacco (Zimmerman and Scorza, 1996) and rice (Park *et al*.,1996). Choice of meristem for transformation also has advantages other than taking relatively less time for transformation *viz*., low somaclonal variation and chromosomal abnormalities and genotype independent.

In the present study also the meristem was used as explant for *Agrobacterium* mediated transformation. Both *in vitro* and *in planta* methods were followed. Use of meristem is especially suitable for transferring any gene causing changes in colour as in the present study transgene itself serving as a reporter.

In two of the 4 cotton genotypes tried for transformation pigment colour changes were observed showing the positive transformation events. Four of 112 established plants of white linted *G. arboreum* genotype PA-255 had purple shade in veins, leaf, squares, bolls and stem (Plate 6a, 6b and 6d). In one of the 4 transformed plant, the petal spot is absent compared to *desi* genotypes with prominent petal spot (Plate 6c). Diploid cotton genotypes inevitably are characterised by dark maroon petal spot and more prominent if the plants are pigmented with reddish colour. However, the transgenic plant in this study though was pigmented in other parts was devoid petal spot indicating the change cause by the transformation. In MB-25, brown linted genotype two out of 117 established plants showed fibre colour change from brown to a shade with sea blue shade (Plate 7a) and one more plant had pink colour which does not exist in the present available colour cotton germplasm. To be able to make textiles of the cotton fibres they must be of good quality which means good strength and length which has to be considered for along with the stability of the colour for ultimately realizing the results of recombinant DNA technology in developing eco-friendly colour cottons.

In earlier reports it has been shown that the action of Cyt P450 enzymes involved in flavonoid synthesis affected flower colour. The hydroxylation of (colourless) dihydroflavonols in the 3' and 5' positions by Cyt P450 enzyme is an important step which determines whether red or purple/blue anthocyanins are formed. In petunia, two loci *hf1* and *hf2*, determine the substitution of anthocyanins in the 3' and the 5' positions resulting in purple and blue flowers. Isolation of the *hf1* and *hf2* loci showed that both encode a Cyt P450 with flavonoid 3',5'-hydroxylase activity (Holton *et al*., 1993). Plant species such as rose and carnation lack F3'5'H activity and are therefore, unable to generate purple or blue flowers. In the present study also the change in the colour was purple as reported in earlier studies rather than perfect blue. This shows that the F3'5'H gene isolated form the petunia when transformed to cotton is able to express. The transformation event has been confirmed through morphological change, PCR amplification of *npt*II F3'5'H gene specific primer.

This study also confirms the usefulness of *in planta* treated meristem explant for *Agrobacterium* mediated transformation.

Future line of work

Isolation and use of a cytochrome b_5 gene reported for full activity of F3'5'H, to achieve the actual blue colour in cotton lint.

The effect of the F3'5'H gene on other important traits especially fiber properties like fibre strength, length and micronaire important for textile industry.

VI. SUMMARY

Cotton is an important fibre crop and a large proportion of cotton cultivated commercially is white. To obtain various shades required by the textile industry white lint need to be dyed with artificial colours. This process is known to use and release affluent that are environmentally hazardous. The naturally coloured cotton would reduce pollution but are restricted due to the availability of just two colours including shades of brown and green. Hence this study focused on the cloning and analysis of flavonoid 3'5' hydroxylase gene responsible for blue shade of petunia flower.

- The petunia genotype with purple colour was used as a source of F3'5'H gene.
- Primers were designed with desired restriction site to isolate the nucleotide segment that encodes F3'5'H gene.
- An amplicon of 2.9 kb was obtained when petunia genomic DNA was subjected to PCR.
- The amplicon cloned in cloning vector was sequenced and the sequence showed 99 per cent homology to the already reported F3'5'H gene (Source: Accession No. AF081575.1 NCBI).
- The nucleotide sequence was replete with three exons and two introns.
- The sequenced gene was subsequently cloned into plant expression vector pHS-100 and the construct pKP1225 was obtained.
- The construct after being transformed into *A. tumafeciens* LBA 4404 was used to genetically engineer the gene into different cotton genotypes.
- Transgenic plants in *G. arboreum* expressing the gene in leaf, veins, stem, squares and bolls were obtained.
- Transgenic plant in *G. hirsutum* expressing the gene in cotton fibre was obtained.

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APPENDIX I

DNA extraction by CTAB method

Extraction buffer

0.35 M Sorbitol

0.1 M Tris-HCl (pH 7.5)

5.0 mM EDTA

20.0 mM SDS

Lysis buffer

0.1 M Tris – HCl (pH 7.5)

20.0 mM EDTA

2.0 M NaCl

55.0 mM CTAB

For extraction buffer (10 ml)

Polyvinyl Pyrrolidone (PVP)	-	100 mg
Dextrose anhydrous AR	-	900 mg
Sodium hydrogen sulphite EP	-	40 mg

APPENDIX II

Components for electrophoresis

a. Loading dye composition

Loading dye (6x)	:	0.25% bromophenol blue
		40% (w/v) sucrose in water

b. Ethidium bromide

10 mg/ml in distilled water. Store at room temperature in dark bottle. Store at 4℃.

c. Recipe for 1 per cent Agarose gel (40 ml)

Agarose	:	400 mg
1x TAE	:	40 ml
EtBr (10 mg/ml)	:	2 µl

d. 50x TAE composition

Tris base	:	242 g
Glacial acetic acid	:	57.1 ml
0.5 M EDTA (pH 8.0)	:	100 ml

Total volume 1000 ml with double distilled water.

APPENDIX III

Conversion table for the amount of a PCR fragment required per ligation reaction

Length of DNA fragment (bp)	Pmoles of ends per 1 μg of DNA	Quantity of PCR fragments for ligation reacting in μg (0.54 pmol ends)
100	30.0	0.018
300	10.0	0.054
500	6.0	0.090
1000	3.0	0.180
2000	1.5	0.360
3000	1.0	0.540

APPENDIX IV

Ligation reaction recipe

Plasmid vector pTZ57R/T DNA (0.165 µg, 0.18 pmol ends)	30 µl
Purified PCR fragment, (Approx. 0.54 pmol ends)	10.0 µl
10x ligation buffer	3.0 µl
PEG 4000 solution	3.0 µl
Deionized water	10.0 µl
T4 DNA ligase, 5U	1.0 µl
Total	30 µl

Control ligation reaction recipe

PTZ57R/T DNA (0.165 µg, 0.18 pmol ends)	3.0 µl
Purified PCR fragment (Approx. 0.54 pmol ends)	12.3 µl
10x ligation buffer	3.0 µl
PEG 4000 solution	3.0 µl
Deionized water upto	29 µl
T4 DNA ligase, 5 U	1.0 µl
Total	30 µl

APPENDIX V

Cloning media

a. Luria agar

Ingredients	Concentration (g/l)
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	5.0
Agar	18.0
pH	7.2

Lauria agar Amp_{50} : To 100 ml Luria agar add 50 µl of Amp_{100} antibiotic at 50℃.

Luria agar Amp_{100} : To 100 ml Luria agar add 100 µl of Amp_{100} antibiotic at 50℃.

IPTG : Dissolve 2 g IPTG in 8 ml water make up volume to 10 ml, filter sterilize, aliquot, store at –20℃

DTT 1M solution : Add 3.09 g DTT in 20 ml of 0.01 M sodium acetate (pH 5.2), store at -20℃

X-gal solution (2% w/v): Dissolve X-gal at concentration of 20 mg/ml in water (Sambrook and Russell, 2001).

b. Recipe for 0.7 per cent Agarose gel (40 ml)

Agarose	:	280 mg
1x TAE	:	40 ml
EtBr (10 mg/ml)	:	2 µl

APPENDIX VI

Reagents for plasmid isolation

STE buffer

Trix-cl (pH 8.0)	: 10 mm
NaCl	: 0.1 M
EDTA (pH 8.0)	: 1.0 mM

Autoclave and store at 4℃

Alkaline lysis solution I

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

Alkaline lysis solution II

NaOH	: 0.2 N
SDS	: 1% (w/v)

(prepare fresh and use at room temperature)

Alkaline lysis solution III

5 M potassium acetate	: 50 ml
Glacial acetic acid	: 11.5 ml
Double distilled water Autoclave and store at 4℃s	: 28.5 ml

APPENDIX VII

Restriction of PCR amplicon and pHS-100 vector

PCR amplicon DNA :	6 µl	
Enzyme Xba I 5 U	:	2 µl
10x buffer	:	2 µl
Sterile water :	10 µl	
Total	:	20 µl

PCR amplicon DNA :	6 µl	
Enzyme BamHI (5U)	:	2 µl
10x buffer	:	2 µl
Sterile water :	8 µl	
Total	:	20 µl

APPENDIX VIII

Ligation of F3'5'H insert and pHS-100

F3'5'H insert	3 µl
Plasmid DNA (pHS-100)	1.5 µl
T_4 DNA ligase enzyme (10 U)	1 µl
Buffer (10 x)	1 µl
Sterile H_2O	3.5 µl
Total	10 µl

APPENDIX IX

Components of Murashige and Skoog (1962) medium

	Components	mg/l concentration
Macronutrients	NH_4NO_3	1650.00
	KNO_3	1900.00
	$MgSO_4 . 7H_2O$	370.00
	KH_2PO_4	170.00
	$CaCl_2 . 2H_2O$	440.00
Micronutrients	$FeSO_4 . 7H_2O$	27.80
	Na_2 EDTA	60.00
	$MnSO_4 . 4H_2O$	22.30
	$ZnSO_4 . 7H_2O$	8.60
	H_3BO_3	6.30
	KI	0.83
	$Na_2MoO_4 . 2H_2O$	0.25
	$CuSO_4 . 5H_2O$	0.025
	$CoCl_2 . 6H_2O$	0.025
Organics	Thiamine HCl	10.00
	Pyridoxine HCl	1.00
	Nicotinic acid	1.00
	Glycine	10.00
	Myoi*nos*itol	100.00
	Biotin	0.50

CLONING AND EXPRESSION OF FLAVONOID 3'5'HYDROXYLASE PIGMENT GENE FOR DEVELOPING ECO FRIENDLY COLOUR COTTON (*Gossypium* spp.)

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ABSTRACT

White cotton lint is dyed and processed to derive various colours. This journey from fiber to fabric is costly and also pollutes the water sources with carcinogenic effluents. The naturally pigmented cottons can reduce the use of dyes. Presently only a few colours like brown and green are available naturally in cotton.

The present study was done to clone flavonoid 3'5'-hydroxylase (F3'5'H), a cytochrome p450 enzyme that catalyses the 3'5'-hydroxylation of dihydroflavonols, the precursors of purple or blue anthocyanins. Genomic DNA of magenta flowered petunia plant was used. Specific primers were designed for the available sequence of F3'5'H for amplification. The 2.9kb amplicon was cloned in cloning vector DH5α and sequenced. The blast results using www.ncbi.nlm.nih.gov showed that the isolated sequence had 99 per cent homology with maximum score bits to two of the seven petunia F3'5'H genes with accession numbers AB 196180.1 and AF081575.1. The isolated gene was subsequently cloned in plant expression vector pHS-100 and the construct pKP1225 was obtained. This was transformed to *Agrobacterium* AH4404 through tri-parental mating with the helper *E. coli* pRK 2013.

The meristem explants of two genotypes (one white linted and one brown linted) in each species of *G.hirsutum* and *G.arboreum* were used for *Agrobacterium* mediated transformation. Both *in planta* and *in vitro* transformation methods were followed. In *in planta* transformation method four out of 112 in white linted genotype, PA-255 had morphological colour change showing purple coloured veins. Two of the 117 in brown linted genotype, MB-25 showed lint colour change. PCR amplification with specific primers confirmed the transformation of F3'5'H gene into cotton. It can be concluded that through PCR using specific primers the F3'5'H gene has been isolated from petunia and is functional as it has expressed in cotton.