“DIFFERENTIAL EXPRESSION OF DFR AND CHI 1 GENES FOR COLOUR MUTANTS IN ONCIDIUM ORCHID”

SUBMITTED BY

Dr. Manju M. George
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DEPARTMENT OF BOTANY
UNION CHRISTIAN COLLEGE
ALUVA, ERNAKULAM (DT.)
KERALA – 683 102

DECEMBER, 2010
PART I: GENERAL INFORMATION

1. Name of the Institute submitting the Project Proposal: UNION CHRISTIAN COLLEGE, ALUVA.

2. State: KERALA.

3. Status of the Institute: (4) State Government Aided Affiliated College (Affiliated to Mahatma Gandhi University, Kottayam)

4. Name and designation of the Executive Authority of the Institute forwarding the application:

   Dr. T. Thomas Philip,
   Principal, Union Christian College,
   Aluva, Ernakulam Dt. Kerala– 683102

5. Project Title: “Differential Expression of DFR and CHI 1 gene for color mutants in Oncidium orchid”

6. Category of the Project: R & D

7. Specific Area: 1.4 Plant Molecular Biology

8. Duration: 3 Years (36 Months)

9. Total Cost (₹): 16,56,200.00
   (₹ Sixteen lakhs Fifty six thousand two hundred only)

10. Is the project Single Institutional or Multiple-Institutional (S/M)? : Single

11. If the project is multi-institutional, please furnish the following: N/A

12. Scope of application indicating anticipated product and processes

Recent advances in genetic engineering and molecular biology techniques augmented with gene transformation could help growers to meet the demand of the orchid industry in the new century. Available methods for the transfer of genes could greatly simplify traditional breeding procedures and overcome some of the inherent genetic problems, which otherwise would not be achievable through conventional methods. Indeed, more recently, orchids have been the subject of new areas of research, including functional genomics, proteomics, and metabolomics. The successful application of these new approaches to improve traits requires a reliable and reproducible transformation technique.
13. **Project Summary.**

Orchids are primarily grown for their large, long-lasting, and fascinating flowers; thus, the improvement of quality attributes such as flower color, longevity, shape, architecture, biotic and abiotic stress tolerance, and creation of novel variations are important economic goals for floriculturists across the world. *Oncidium* (Dancing girl/doll) is a commercially important orchid, and considerable research efforts of numerous biotechnologies have been deployed in the past few years to improve its mass propagation, gene transformation and disease resistance. The ability to regenerate plants from cultured cells, tissues or organs provides an efficient tool for plant transformation. Protocorm-like bodies (PLBs) were the most convenient target tissue in different gene transformation systems including *Agrobacterium*-mediated and micro projectile bombardment. A range of available molecular and biochemical tools to confirm transgene integration and expression made the outcome of such experiments more understandable. The integrated approaches of genetic engineering in *Oncidium* have been of great value because of its commercial value. Indeed, more recently *Oncidium* is the subject of new areas of research including functional genomics, proteomics, and metabolomics. For the successful application of these new approaches to improve traits in *Oncidium* a reliable and reproducible transformation technique is essential.

The aim of the present study is to transform an important orchid *Oncidium* for developing color mutants. The genes we selected were DFR (dihydroflavanol 4-reductase) and F3H1 (flavanone 3- hydroxylase) gene isolated from Soybean plants. The Constructs available are cloned in *Agrobacterium tumefacians* for Co culture.

1. LBA 4404 - pLMG - F3H1 – GUS (vector pCambia 1301)  
2. LBA 4404 – pLMG - DFR1- GUS (vector pCambia 1301)

PLB (protocorm like body) of *Oncidium* derived from stem nodes or buds will be used as the source for transformation studies. PLB were subculture from liquid/solid medium to new media plates before 1 week later Chop the PLB to 2-3mm size and precultured for 3-5 days. In the modified method new media will be used for co cultivation and subsequent studies. To enhance the cocultivation efficiency alteration in the growing media will be done. The NH$_4$NO$_3$ was removed from the media ingredients in my earlier studies has some positive results. In the present study I would like to continue the study and will try to develop an high efficiency transformation system in *Oncidium*. 
PART II: PARTICULARS OF INVESTIGATOR.

Principal Investigator:

14. Name: Dr. Manju M. George  
   Date of Birth: 23/11/1971        Sex (M/F) : Male.

   Designation: Assistant Professor.

   Department: Botany.

   Institute/University: Union Christian College.

   Address: Union Christian College, UCC Post, Aluva, Kerala - 683102

   Telephone: 0484-260-9194        Fax:0484-2607534,  0484-2477002 (res)

   E-mail: manjumgeorge@uccollege.edu.in  
           meluttu@gmail.com

   Number of Research projects being handled at present: One UGC Minor Project  
                                                      (1-1-10 to 31-12-10)

Co-Investigator

15. Name: Nil
16. Introduction
Orchidaceae is one of the largest families of flowering plants, which consists of more than 800 genera and 25,000 species of which many are of commercial importance. Traditional breeding methods (i.e. continuous crossing and selection) have paved the way for the breeders to create new varieties that have desirable traits viz. color, shape, fragrance, plant architecture, vase life and resistance to disease and pests but this kind is the limited gene pool of any single species. Millions of seeds are formed in a single orchid capsule but didn’t have any metabolic machinery and hence no endosperm is developed fully. Only few seeds germinate in nature from the large number of seeds produced. Currently, the horticultural trade depends on wild orchid population as a source of stock plants, but most are not propagated commercially. *Oncidium* sp., one of the commercially important orchids, is selected for this study because it produces a brightly attractive flowers and it is highly adaptable to culture under a wide range of climatic conditions.

Molecular approaches have opened a new way for the genetic transformation of plants to create novel traits, such as flower color modulations, floral formation, plant and inflorescence architecture, fragrance etc. The creation of mutant cultivar has become an important factor in the market for commercial orchid production. Novelty is the driving force in ornamental plant industry. Hence molecular genetic modification can be adopted in addition to traditional breeding for the emergence of plants with novel aesthetic properties.

In orchids protocorms from germinated seeds or protocorms-like-bodies (PLBs) derived from shoot tip or leaves are the most easily obtained materials that are capable of regenerating plants. The routine transformation procedure for orchids via either *Agrobacterium*-mediated or microprojectile bombardment for introducing genes with horticultural and economically important traits, such as virus disease resistance, is being started. Several studies on successful transformation of *Phalaenopsis*, *Oncidium*, *Cymbidium*, and *Dendrobium* have been reported. However, long period of selection and regeneration, and low recovery of transgenic plants have hindered the efficient transformation of these recalcitrant orchid species.

16.1 Origin of the proposal

**Targets for molecular flower breeding of orchids**

**Flower color modification**

Flower color of higher plants is due to the production of pigments, including flavonoids, carotenoids, and betalains. Flavonoids contribute wide spectrum of colors in plants, including red, blue, yellow and purple pigments. Six subgroups of the flavonoids are
widespread in plants, including chalcones, flavones, flavonols, flavandiols, anthocyanins, and condensed tannins (or proanthocyanidins). The biosynthesis of anthocyanin pigments and flavonol co pigments made flowers showy and function to recruit pollinators and seed dispersers. Direct modification of anthocyanin production has been achieved in several plant species, such as petunia and carnation. Wild type carnation was modified to produce blue colors after introducing a heterologous flavonoid 3',5'-hydroxylase gene. Transgenic carnation with selected color shades has been introduced in Japan market. This points the possibility of color modification through gene technology in the non-food flower crops such as orchids.

Transgenic plants are produced via Agrobacterium-mediated and other direct DNA transfer methods like PEG, electroporation, microprojectile bombardment, and microinjection. In orchids protocorms from germinated seeds or protocorms-like-bodies (PLBs) derived from shoot tip or leaves are the most easily obtained materials that are capable of regenerating plants. The routine transformation procedure for orchids via either Agrobacterium-mediated or microprojectile bombardment for introducing genes with horticultural and economically important traits, such as virus disease resistance, is being started. Several studies on successful transformation of Phalaenopsis, Oncidium, Cymbidium, and Dendrobium have been reported. A high efficient Orchid transformation is important not only for inserting genes for improving the crop traits but also for basic research studies that is the basis for future plant improvement. The various genome initiatives have generated a large number of genes that should be tested in Orchid plants to study the gene function. Hence this newer methods will simplify the Orchid transformation for insertion of desired foreign genes in to this important plant and further studies.

16.2 (a) Rationale of the study supported by cited literature
Few papers have been published on Oncidium transformation through Agrobacterium mediated methods (Liau et al 2003 a,b; You et al, 2003). They reported about incorporation of reporter genes GUS and PFLP (Lin, 1997) which confers resistance to soft rot diseases in Oncidium. The addition of tobacco suspension culture as nurse culture was helpful for the transformation of this orchid. They used a two step method in acidic pH (5.4) used to induce agro for its vir gene induction. A slow selection procedure is applied at first month by addition of timentin and cefotaxime to kill the bacteria and Hygromycin only in the second month for selection of transformed tissues. Hence they concluded a long period at low selection pressure is essential for successful transformation. Similar reports were also published in Dendrobium transformation by Yu et al (2001).
Methods to enhance Agrobacterium-mediated transformation.

In recent years various approaches were examined for improvement of transformation efficiency in various plant species. Roberts et al (2003) used purine synthesis inhibitors to cause super sensitivity to Agrobacterium tumefaciens in three plant species. Hoshi et al (2004) developed an efficient method of transformation by a scratching treatment of calli before co-cultivation and eliminating a major macronutrient ammonium nitrate (NH$_4$NO$_3$) from the culture medium. The response of plants to wounding is an important factor in the interaction between Agrobacterium and plants. (Potrykus, 1991). Since the amount of Agrobacterium that proliferated over and around the calli during co-cultivation increased as the concentration of NH$_4$NO$_3$ decreased, the inhibitory effect of NH$_4$NO$_3$ on the transient transformation efficiency might result from the inhibition of the proliferation of Agrobacterium by NH$_4$NO$_3$ (Hoshi et al, 2004).

In rubber tree Montoro et al (2003) reported the use of calcium free media, both in bacteria and plant tissue culture preparations dramatically increased the number of transformation events. Usually cocultivation is done in dark for 2-3 days in many of the transformation studies. Here they reported incubation of two plant species Phaseolus and Arabidopsis in continuous light or 16/8 photoperiod during cocultivation period and found a substantial increase in T-DNA transfer. A combination of the above methods or alone can improve the transformation efficiency in Orchids too.

A high efficient Orchid transformation is important not only for inserting genes for improving the crop traits but also for basic research studies that is the basis for future plant improvement. The various genome initiatives have generated a large number of genes that should be tested in Orchid plants to study the gene function. Hence this newer methods will simplify the Orchid transformation for insertion of desired foreign genes in to this important plant and further studies.

(b) Hypothesis

- Rapid propagation of Oncidium orchids is possible by production of PLB’s.
- The rate of transformation can be increased by altering the media components.
- Color mutants can be produced in Oncidium orchid of Ornamental value by transferring pigment genes from Soybean.

(c) Key questions.

- Rapid propagation of Oncidium orchids is possible or not?
- Can we enhance the rate of transformation by altering the media components?
Can we produce color mutants of Ornamental value using pigment genes from Soybean?

16.5 Current status of research and development in the subject (both international and national status)

Extensive research on Orchids is being carried out especially in Asian Countries like Taiwan and Japan. In National Level most of the research is being carried out by Institutes in Northeastern states However basic research are being carried out in Kerala Agricultural University, Tropical Botanical Garden, Palode and other few institutes. There are many Private companies engaged in Cutflower business of Orchids flowers mainly AVT Biotech, Hafi Biotech, Shobha Orchids etc. Extensive molecular level research is less in India compared to Taiwan and Japan where they do research on all aspects of Orchid research.

16.6 The relevance and expected outcome of the proposed study

A high efficient rapid propagation of Oncidium Orchids can be standardized. The cut flower business in Cochin area is increasing and Orchids have high importance. Oncidium is widely cultivated in the local climatic conditions but only one variety is available locally. If a color mutant can be available it will be of high economic importance with respect to Ornamental Horticulture business in Kerala which is booming like anything in the present scenario.

16.7 Preliminary work done so far

In the short term study I could observe that PLB’s of Oncidium Orchids can be induced from stem nodal explants and it can be subculture and maintained with regeneration potential up to 5-6 subcultures. Multiple shoots can be induced from the same and these PLB’s can be used for transformation studies. Regarding the transformation studies when Ammonium nitrate has been removed form the plant culture medium; it has helped in the Cocultivation stages. A clearly visible growth of Agrobacterium is seen around the PLB after three days of co cultivation while in Normal media this was not observed. Further detailed study has to be done in the present study.

17. Specific objectives

Objectives:

- Collection and Maintenance of Healthy Plants
- Select the suitable explants for the induction of protocorm like bodies (PLB’s)
- Multiplication and maintenance of PLB’s
- Growing Agrobacterium tumefaciens LBA4404 with desired genes.
- Agro infection by co culturing for gene transfer
• Selection of transformed tissues
• Regeneration of Transformed Plantlets
• Confirmation for Transient/Transgenic expression
• Raising more transformed plantlets
• Acclimatization of transformed plantlets
• Flower analysis for color mutants
• Raising more color mutants

18. Work Plan:

18.1 Work plan (methodology/experimental design to accomplish the stated aim)

1) Methodology for Tissue Culture propagation of Oncidium.

Plant Selection: The Oncidium plants will be collected and grown in poly house and will be propagated.

Explants:

(i) Stem Nodal explants will be selected for the induction of protocorm like bodies (PLB’s).

(ii) The green pods still containing the dry petals at their tips will be collected from grown plants and used for embryo culture. First the dry petals attached to the green pods were removed, then the pods were washed thoroughly using running tap water.

The pods and stem nodal explants were surface sterilized by treating with 1-5% Sodium hypochloride solution for 20-30 min followed by thorough wash in sterile double distilled water.

Media:
Murashige and Skoogs (1962) media with 2mg/L BA and 1mg/L NAA were found to be good for induction of PLB’s and for subculturing and maintaining the PLB’s. For altering the media for enhancing the transformation rate NH₄NO₃ will be eliminated from MS media.

2) Agrobacterium construct and details

Agrobacterium tumefaciens strain LBA 4404 harbouring the plasmid pCAMBIA 1301 with the β-glucuronidase (GUS) gene interrupted with a plant intron (GUS-INT) (Vancanney et al., 1990) driven by the Cauliflower Mosaic Virus 35S promoter (CaMV 35S) and nopaline synthase terminator as reporter gene was used for transformation. This vector has hygromycin phosphotransferase (hptI) gene in T- DNA region driven by CaMV35S promoter and CaMV35S polyA terminator confers resistance to the antibiotic hygromycin as a plant selection marker and neomycin phosphotransferase (nptII) gene driven by the CaMV35S
promoter and nopaline synthase (NOS) terminator outside to T-DNA region, which confers resistance to the antibiotic kanamycin as a bacterial selection marker.

3) Methodology for *Agrobacterium tumefaciens* LBA4404 growth.

Streaked single colony from agar plates to 5 ml culture with Strep and Kan for 2 days then 1 ml to 50ml culture from the above with Strep (25mg/L) and Kan (50mg/L)

4) Sub culturing and multiplication of *Oncidium*.

**Media:**
Murashige and Skoogs (1962) media with 2mg/L BA and 1mg/L NAA were found to be good for subculturing and maintaining the PLB’s.

5) Transformation Protocol.

**Pre-conditioning of PLB’s**
PLB will be subcultured from medium to new MS plates before 1 week, later Chop the PLB to 2-3mm size and preculture for 3-5 days. In the modified method MS-1 media were used for co-cultivation and subsequent studies. The Ammonium nitrate will removed from the media ingredients.

**Procedure**

Via *Agrobacterium*. Streaked single colony from agar plates to 5 ml culture with Strep and Kan for 2 days then 1 ml to 50ml culture from the above with Strep (25mg/L) and Kan (50mg/L) for overnight. 200 µM Acetosyringone (AS) will be added to media and incubate in shaker for 4 hours before the experiment. Centrifuge the bacteria and dissolve in 50 ml MS basal media. The pretreated PLBs were dipped in the above 10 ml culture for 30 minutes, later dried in sterile filter paper and kept 3 days in co-cultivation media (MS + AS-400µM) in complete dark. Later washed with sterile water 2-3 three times depending on the overgrowth and with Cefotaxime (400mg/L).

6) Selection.

Plated in Selection media first without any selection pressure but only with cefotaxime or Timentin (250mg/L) to kill the bacteria and after two weeks in lower concentration (2mg/L) Hygromycin + 250mg/L Cefotaxime. Subculture every week or depends on the Agro growth and increase the Hygromycin (to 5 mg/L) concentration after two weeks.

Method by Liau etal (2003) also will be tried.

-A single colony of Agro containing the construct will be transferred in to 5 ml AB medium containing 100mg/L Kanamycin incubated at 28°C for 2 days

-The bacterial culture will then transferred to 50 ml AB medium containing 100 mg/L Kan and incubated at 28°C for overnight

10
-about 4 hrs before transformation 200 µM AS will be added to the bacterial culture

-The agro culture (OD 0.8-1) will be centrifuged at 3,000 g for 10 min and the pellet will suspended in 20 ml MS medium

-About 15 ml culture was added to each of the preculture plates containing PLB’s and incubated at RT for 30 min

-PLB’s were blotted dry on sterile filter paper and co-cultivated on G10 medium plates without charcoal but with 200 µM AS and a nurse culture of tobacco suspension cells which were added to the medium when warm (37°C) and incubated at 25°C in darkness for 3 days.

-The infected PLB’s were washed with MS medium containing 200mg/L timentin to prevent Agro overgrowth and then transferred to G10 medium supplemented with 100 mg/L timentin and 50mg/L Cefotaxime

-The PLB’S were cultured with 16 Hr photoperiod at 25°C and subculture every week for one month

-transformed PLB were transferred to a PR medium with 5 mg/L hygromycin for selecting putative transformants

-The newly formed green PLBs were further subcultured to fresh G10 medium every month for 3 months.

After 3 months PLB’S were subcultured on G10 medium without hygromycin for root formation.

G10 medium:- MS salts 1g/L tryptone, 20g/L Sucrose, 1g/L charcoal, 65g/L potato, 3 g/L phytagel.
AB medium:- KH2PO4 7.25, K2HPO4 10.25 g/L, NaCl 0.15 g/L, CaCl2 0.01 g/L, Glucose 5g/L, FeSo4 2.5mg/L Agar 8g/L

7) Regeneration of Transformed Plantlets
The transformed plants selected by the above methods will be selected and analysed till flowering.

8) Molecular analyses of transgenic plants
Primers for DFR, F3H1, hptII, GUS will be made and used for PCR
The genomes of the independent GUS-positive, resistant transgenic lines will be analyzed for transgene integration by Southern hybridization.
Total genomic DNA, isolated from the leaves of the transformed and WT plants, was digested with BamHI/HindIII and probed with the DFR/F3H1 gene.

9) Acclimatization of normal/transformed plantlets
Hardening:
The Healthy well-developed seedling 2-5 cm height will be removed from culture vessels and thoroughly washed with tap water to remove adhering medium completely without
causing damage to the roots. Then the plantlets will be treated with the fungicide solution (Bavistin) at low concentration and transferred to perforated plastic pots. Plastic pots will be filled with a mixture of uniform, small charcoal pieces and brick pieces (1:1). After a thorough wash of the pots and the potting media in water and treatment with any fungicide, the seedling would be transplanted. Care should be taken so that 1-2 roots of the seedlings passed through the space in between charcoal pieces. The potted plants will be kept under a green house (20-30 % light) and mist irrigated. After two weeks both misting and foliar application of NPK mixture were followed. The application of the later was done twice in a week. Observations on the establishment of the seedlings will be recorded at regular intervals.

10) Flower analysis for color mutants & Raising more color mutants

**Anthocyanin analysis**
The extraction of anthocyanin pigments will be carried carried out following the method (Goodman et al. 2004).

*Oncidium* floral tissues were extracted by grinding in appropriate solvent of 0.1 N HCl in methanol. The ground tissues will be immediately centrifuged to separated debris for twice. The supernatant was removed, and diluted with 5% acetic acid in ratio ranging from 1:1 to 20:1 depending on the pigment concentration. The final solution will be applied to HPLC analysis immediately.

18.2 Connectivity of the participating institutions and investigators (in case of multi-institutional projects only)

N/A

18.3 Alternate strategies (if the proposed experimental design or method does not work what is the alternate strategy)

Since the preliminary work has already done I believe it will work out. If not the efficiency of *Agrobacterium* mediated transformation is less I will go for an alternative method of Gene transformation by particle gun (microprojectile bombardment) which is not available in the parent institute but will seek help from other institutes.
19. **Timelines**: (Please provide quantifiable outputs)

<table>
<thead>
<tr>
<th>Period of study</th>
<th>Achievable targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Months</td>
<td>Lab Augmentation, Collection and multiplication of plants, Collection of research articles, Initiation of tissue culture methods.</td>
</tr>
<tr>
<td>12 Months</td>
<td>Standardization of protocol for rapid multiplication through PLB’s, <em>Agrobacterium</em> co-cultivation and infection</td>
</tr>
<tr>
<td>18 Months</td>
<td><em>Agrobacterium</em> co-cultivation and infection, Tissue culture Multiplication and standardisation</td>
</tr>
<tr>
<td>24 Months</td>
<td><em>Agrobacterium</em> co-cultivation and infection. Analysis for transient/transgenic expression of color mutant genes</td>
</tr>
<tr>
<td>30 Months</td>
<td>Analysis for transient/transgenic expression of color mutant genes</td>
</tr>
<tr>
<td>36 Months</td>
<td>Quantification, standardization of transformed plants. Acclimatization of Color mutant plants, Hardened plants Released outside the greenhouse</td>
</tr>
</tbody>
</table>

20. **Name and address of 5 experts in the field**

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Name</th>
<th>Designation</th>
<th>Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dr. Fure Chy Chen</td>
<td>Professor</td>
<td>Department of Plant Industry National Pingtung University of Science &amp; Technology, Nei-pu, Taiwan</td>
</tr>
<tr>
<td>2</td>
<td>Dr. R B Subramanian</td>
<td>Assoc. Prof</td>
<td>BRD School of Biosciences Sardar patel University Vallabh Vidy anagar Anand, Gujarat - 388120</td>
</tr>
<tr>
<td>3</td>
<td>Dr. Krishanan Kathiravan</td>
<td>Assoc. Prof</td>
<td>Department of Biotechnology University of Madras Chennai, Tamil Nadu</td>
</tr>
<tr>
<td>4</td>
<td>Dr. J S.S Mohan</td>
<td>Assoc. Prof</td>
<td>BRD School of Biosciences Sardar patel University Vallabh Vidy anagar Anand, Gujarat - 388120</td>
</tr>
<tr>
<td>5</td>
<td>Dr. P. K. Rajeevan</td>
<td>Professor</td>
<td>Professor &amp; Head Dept of Floriculture &amp; Pomology, Kerala Agricultural University, Vellanikkara, Mannuthy, Trichur, Kerala</td>
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**PART IV: BUDGET PARTICULARS**

**Budget (In Rupees)**

A. Non-Recurring (e.g. equipments, accessories, etc.)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Item</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
<th>Total (₹)</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>Hybridization Oven</td>
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<td>2.</td>
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<td>3.</td>
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Sub-Total (A) = 5,27,000.00

B. Recurring

B.1 Manpower (See guidelines at Annexure-III)

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<tr>
<th>S. No.</th>
<th>Position No.</th>
<th>Consolidated Emolument</th>
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<th>Year 2</th>
<th>Year 3</th>
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<td>One Junior Research Fellow</td>
<td>12,000 (first 2 years) 14,000 (3rd year)</td>
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<td>144,000</td>
<td>168,000</td>
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Sub-Total (B.1) = 4,56,000.00

B.2 Consumables

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<th>Year 2</th>
<th>Year 3</th>
<th>Total (₹)</th>
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<td>Glass ware</td>
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Sub-Total (B.2) = 2,70,000.00

Other items

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<th>Consolidated Emolument</th>
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<th>Year 2</th>
<th>Year 3</th>
<th>Total (₹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.3 Travel</td>
<td>--</td>
<td>15,000</td>
<td>10,000</td>
<td>10,000</td>
<td>35,000</td>
</tr>
<tr>
<td>B.4 Contingency</td>
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<td>80,000</td>
<td>60,000</td>
<td>40,000</td>
<td>1,80,000</td>
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<tr>
<td>B.5 Overhead @ 20% of total cost of recurring</td>
<td>188,200</td>
<td>188,200</td>
<td>--</td>
<td>--</td>
<td>1,88,200</td>
</tr>
<tr>
<td>Sub-total of B (B.1+B.2+B.3+B.4+B.5)</td>
<td>--</td>
<td>369,000</td>
<td>294,000</td>
<td>278,000</td>
<td>11,29,200</td>
</tr>
<tr>
<td>Grand Total (A + B)</td>
<td>--</td>
<td>10,84,200</td>
<td>2,94,000</td>
<td>2,78,000</td>
<td>16,56,200</td>
</tr>
</tbody>
</table>

Grand Total: ₹ 16,56,200/- (Rupees Sixteen lakhs Fifty six thousand two hundred only)

**Note:** Please give justification for each head and sub-head separately mentioned in the above table.

Financial Year: April - March
**Justification for budget particulars:**

A.1 Hybridization Oven is essential for incubating the nylon membranes at particular temperature in southern and northern blots for molecular analysis of transformed plants and for other hybridization works.

A.2 Micropipettes are needed for the use of growth hormones and other chemicals in minute quantity and for accuracy. 5 pipettes are needed at varying ranges. (0.1-1.0 µl, 1-2 µl, 1-10 µl, 10-100 µl, 50-200 µl, 500-5000 µl)

A.3 For the storage of chemicals, Media, Growth hormones etc

A.4 For the storage of Growth Hormones, Enzymes and other chemicals a deep freezer is very much essential

A.5 Bacterial Incubator is needed for the growth of *E.coli* and *Agrobacterium* for the project work.

A.6 Hotplate with magnetic stirrer is needed for mixing media and other chemicals.

A.7 Books and Journals are needed for the updated references for both PI and Research fellow.

A.8 A desktop/laptop PC and printer is needed for documentation, storage of information’s, Search the e-libraries, collection of research articles, etc for the proposed research work.

B.1 Manpower. A junior research fellow is very much needed to carry out the research work along with the PI.

B.2 Consumables.

   **Chemicals:** Chemicals, growth hormones, enzymes etc are needed for the Research work, For the preparation of media, chemical analysis etc.

   **Glasswares:** Glassware’s are needed for the preparation of media, storage and for Culture Incubation, etc.

B.3 Travel grant is needed for collection of plant materials, to carry out reference work at various institutes like TNAU, IISc etc and also to present the findings in various seminars and symposia.

B.4 Contingency grant is needed for the purchase of stationary items including paper, printing, Xerox etc. Accessories to equipments, pipette tips, maintenance of Equipments and any other special needs other than specified in other budget items.

B.5 Overhead cost @ 20% of consumable items is needed for the host institute for Administrative purpose, electricity charges etc.
PART V : EXISTING FACILITIES

Resources and additional information

1. Laboratory:
   a. Manpower

   So far no manpower is there to conduct the research activities. Hence we are proposing a JRF in the project to carry out the research work.

   b. Equipments

   ✓ A well furnished lab,
   ✓ Tissue culture room with racks,
   ✓ 2 Laminar Airflow Bench,
   ✓ 3 Autoclaves,
   ✓ PCR,
   ✓ Electrophoresis units,
   ✓ Glass Double Distillation Unit
   ✓ Refrigerator
   ✓ Camera,
   ✓ LCD projector etc

2. Other resources such as clinical material, animal house facility, glass house. Experimental garden, pilot plant facility etc.

   ✓ Two small net houses are available for the growth of plants.
   ✓ Botanic Garden and Arboretum maintaining by the Department of Botany.
PART VI: DECLARATION/CERTIFICATION

It is certified that

a) the research work proposed in the scheme/project does not in any way duplicate the work already done or being carried out elsewhere on the subject.

b) the same project proposal has not been submitted to any other agency for financial support.

c) the emoluments for the manpower proposed are those admissible to persons of corresponding status employed in the institute/university or as per the Ministry of Science & Technology guidelines (Annexure-III)

d) necessary provision for the scheme/project will be made in the Institute/University/State budget in anticipation of the sanction of the scheme/project.

e) if the project involves the utilisation of genetically engineered organisms, we agree to submit an application through our Institutional Biosafety Committee. We also declare that while conducting experiments, the Biosafety Guidelines of the Department of Biotechnology would be followed in toto.

f) if the project involves field trials/experiments/exchange of specimens, etc. we will ensure that ethical clearances would be taken from concerned ethical Committees/Competent authorities and the same would be conveyed to the Department of Biotechnology before implementing the project.

g) it is agreed that any research outcome or intellectual property right(s) on the invention(s) arising out of the project shall be taken in accordance with the instructions issued with the approval of the Ministry of Finance, Department of Expenditure, as contained in Annexure-V.

h) we agree to accept the terms and conditions as enclosed in Annexure-IV. The same is signed and enclosed.

i) the institute/university agrees that the equipment, other basic facilities and such other administrative facilities as per terms and conditions of the grant will be extended to investigator(s) throughout the duration of the project.

j) the Institute assumes to undertake the financial and other management responsibilities of the project.

Signature of Executive Authority of Institute with seal

Date : 27-12-2010 Principal

Signature of Principal Investigator: Seal
Date : 27-12-2010
PART VII:

PROFORMA FOR BIOGRAPHICAL SKETCH OF PRINCIPAL INVESTIGATOR

Name: Dr. Manju M George
Designation: Assistant Professor in Botany
Department/Institute/University: Union Christian College, Aluva 683102
Date of Birth: 23/11/1971 Sex (M/F) Male SC/ST: NA

Education (Post-Graduation onwards & Professional Career)

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Institution and Place</th>
<th>Degree Awarded</th>
<th>Year</th>
<th>Field of Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dept. of Biosciences Sardar Patel University Vallabhbh Vidyanagar.</td>
<td>M.Sc</td>
<td>1995</td>
<td>Botany</td>
</tr>
<tr>
<td>2</td>
<td>Dept. of Biosciences Sardar Patel University Vallabhbh Vidyanagar</td>
<td>Ph.D</td>
<td>2000</td>
<td>Botany (Specialization-Plant Biotechnology)</td>
</tr>
<tr>
<td>3</td>
<td>NIFTS, Japan NPUST, Taiwan KGTRI, S.Korea UoMN, USA</td>
<td>PDF</td>
<td>2001-2007</td>
<td>Plant Biotechnology</td>
</tr>
</tbody>
</table>

A. Position and Honors

B. Position and Employment (Starting with the most recent employment)

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Institution and Place</th>
<th>Position</th>
<th>From (Date)</th>
<th>To (date)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Union Christian College Aluva, Kerala</td>
<td>Asst. professor in Botany</td>
<td>27-7-2006</td>
<td>Till Date</td>
</tr>
<tr>
<td>3</td>
<td>Department of Biosciences, Sardar Patel University, India.</td>
<td>Research Associate</td>
<td>15-6-2005</td>
<td>31-5-2006</td>
</tr>
<tr>
<td>4</td>
<td>Department of Plant Industry, National Pingtung University of Science &amp; Technology, Nei-pu, Taiwan</td>
<td>Post doctoral fellow</td>
<td>Jan, 2004</td>
<td>May 2005</td>
</tr>
<tr>
<td>5</td>
<td>Department of Horticulture, University of Minnesota, St.Paul, USA</td>
<td>Post Doctoral Associate</td>
<td>March 2002</td>
<td>Feb 2003</td>
</tr>
<tr>
<td>6</td>
<td>Korea Ginseng and Tobacco Research Institute, Taejon, South Korea</td>
<td>Post Doctoral fellow</td>
<td>Feb 2001</td>
<td>Jan 2002</td>
</tr>
<tr>
<td>7</td>
<td>Department of Biosciences, Sardar Patel University, India</td>
<td>Research Fellow</td>
<td>Apr 1996</td>
<td>Jan 2001</td>
</tr>
</tbody>
</table>
Honors/Awards
1. Inoue Young Scientist Award for Research in NIFTS, Japan by Inoue Foundation for Science,(IFS, JAPAN) 2007
3. Selected as Project fellow in UGC sponsored major research project entitled “Improvement of essential oil content in lemongrass (Cymbopogon sp.) and Vetiver through in vitro techniques” for three years.
4. Received merit scholarship for Ph.D. in Botany from Sardar Patel University

Professional Experience and Training relevant to the Project

Doctoral work in India and Post Doctoral Works at various foreign institutions area in the area of plant Biotechnology.
Research work in NPUST, Taiwan:-
Specifically worked on a project to develop transformation protocols of Oncidium transformation for tagging important genes affecting developmental character such as flowering. I tried to obtain transgenic Oncidium in the first stage and then use different vector constructs to transform the orchid. I had established enough protocorm-like bodies (PLBs) for transformation experiments from the shoot node. I have used A.tumefaciens LBA4404 and particle gun (Biorad) for transformation of orchids. So far I have transformed enough PLB with DFR – (dihydroflavanol 4-reductase) F3H1 –( flavanone 3-hydroxylase) genes. (Both soybean pigment genes which can induce color mutations). The vector used is pCAMBIA 1301 and 1305.1 as control. PLB were transformed with both Agrobacterium and gene gun. GUS staining confirmed transformation with all constructs. From selected transformants shooting from the PLB and further analysis were done.

B. Publications (Numbers only)
Books : Nil      Research Papers, Reports 8      General articles . 1..
Patents : Nil      Others (Please specify) : Nil

Selected peer-reviewed publications (Ten best publications in chronological order)
4) Deok-Chun Yang, Manju Meluttu George, Jong-Seong Jeon (2003) In vitro propagation of Coleus forskohlii, an important medicinal plant. Plant Resources. 6 (2) 129-133
6) Manju M.George and R.B.Subramanian (2000) Regeneration of West Indian Lemongrass Cymbopogon citratus DC (stafp) (Citrongrass) from immature
List maximum of five recent publications relevant to the proposed area of work.
1) Genetic transformation of Oncidium orchid using protocorm-like bodies (PLBs). (2009: Plant Cell Biotechnology and Molecular Biology) In press:
4) Deok-Chun Yang, Manju Meluttu George, Jong-Seong Jeon (2003) In vitro propagation of Coleus forskohlii, an important medicinal plant. Plant Resources. 6 (2) 129-133

C. Research Support
Ongoing Research Projects

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Title of Project</th>
<th>Funding Agency</th>
<th>Amount</th>
<th>Date of sanction and Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Transformation studies in Coleus forskohlii an important medicinal plant.</td>
<td>UGC</td>
<td>145,000</td>
<td>1-1-2010, One Year UGC Minor project</td>
</tr>
</tbody>
</table>

Completed Research Projects (State only major projects of last 3 years)

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Title of Project</th>
<th>Funding Agency</th>
<th>Amount</th>
<th>Date of completion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1. Approval of the Research proposal and the grant released would be for the specific project mentioned in paras I to V of this proposal and grant should be exclusively spent on the project for which it has been sanctioned within the stipulated time. The Institute is not permitted to seek or utilize funds from any other organization (Government, Semi Government, Autonomous or Private) for this research project. Any unspent part of amount would be surrendered to the Govt. of India through an account payee demand draft drawn in favour of the “Drawing and Disbursing Officer, Department of Biotechnology, New Delhi”, and carry forward of funds of the next financial year for utilization for the same project may be considered only with the specific approval of the Department of Biotechnology (DBT).

2. For permanent/semi-permanent assets acquired solely or mainly out of the grant, an audited record in the form of a register in the prescribed proforma (enclosed at Appendix-'A') shall be maintained by the Institute. The term “assets” means (I) immovable property and (II) movable property of a capital nature, where the value exceeds Rs. 1000/- The grant will not be utilised for construction of any immovable property, Full facilities by way of accommodation, etc. for the project will be given by the Institute.

3. All the assets acquired from the grant will be the property of Govt. of India and should not without the prior sanction of the Deptt. of Biotechnology, be disposed of, or encumbered or utilised for purpose other than those for which the grant has been sanctioned.

4. At the conclusion of the project, the Govt. of India will be free to sell or otherwise dispose of assets which are the property of the Government. The Institute shall render to Govt. necessary facilities for arranging the sale / disposal of these assets. The Government may, however, consider the request of host institutions to retain the assets created under a project for carrying out similar work for the promotion of science.

5. The implementing Institute/PI will furnish progress report of work on the project every six months. The progress of the project will also be reviewed/monitored at least once a year by the concerned Task Force/Project Monitoring Committee, etc. In addition the DBT shall designate Scientists/Specialists to visit the Institute periodically for reviewing the progress of work and for suggesting such measures as to ensure early realisation of the objectives of the project. On completion of the project five copies of a consolidated report of the work done on the subject would be submitted to the Department of Biotechnology.

6. The Institute is required to send to DBT a list of assets referred to at Sl. No. 2 above at the end of each financial year as well as at the time of seeking further installments of the grant.

7. The Institute would furnish to the Deptt. of Biotechnology a Utilization Certificate (Copy enclosed at Appendix - 'B’) and an audited statement of expenditure (Copy enclosed at Appendix - ‘C’) duly signed by the P.I., the Head of the Institute and the
Head of the Finance wing, pertaining to the grant at the end of each financial year as well as a consolidated statement of expenditure at the end of the completion of the project.

8. A stamped receipt be sent to the Deptt. of Biotechnology on receipt of the Cheque/Demand draft towards each release.

9. The Comptroller and Auditor-General of India at his discretion shall have the right of access to the books and accounts of the Institute for the grant received from the Government.

10. The Institute would maintain separate audited accounts for the project. If it is found expedient to keep a part or whole of the grant in a bank account earning interest, the interest thus earned should be reported to the Deptt. of Biotechnology.

11. Sale proceeds, if any, as a result of the development of the project arising directly from funds granted by the Deptt. of Biotechnology shall be reported to the Govt. of India. The Govt. of India may at its discretion allow a portion of such receipt to be retained by the Institute for its utilisation for the project activities.

12. Investigators/Institutes wishing to publish papers based on the research work done under Deptt. of Biotechnology projects should acknowledge the financial support received from the Deptt. of Biotechnology.

13. Investigators/Institutes may utilize various resources such as the Bioinformatics resources, experimental materials, reagents, cell lines, animals, etc. from the National facilities/Institutes/Centres established by this Department as per the terms of transactions followed by them. More information may be obtained about such facility from DBT websites: www.dbtindia.org / www.dbtindia.nic.in, www.btisnet.ac.in.

14. Investigators / Institutes shall follow the detailed instructions on technology transfer and Intellectual Property Rights (IPR) as given at Annexure - V. The same has the approval of the Ministry of Finance, Govt. of India vide Deptt. of Expenditure, Plan Finance II – Division Letter No. 33 (5) /PF.II/99 dated 22nd February, 2000. Any deviation from these instructions may be brought to the notice of this Department.

15. Investigators / Institutes may file patents with the help of the Biotechnology Patents Facilitating Cell (BPFC) established at DBT on priority bases. The format for filing the patents may be seen at Annexure –VI.

16. The Govt. of India (Deptt. of Biotechnology) will have the right to call for drawings, specifications and other data necessary to enable the transfer of know-how to other parties and the Institute shall supply all the needed information at the request of the Department of Biotechnology which will ensure confidentiality. The information required for commercializing Biotechnologies may be furnished to this Deptt. as per the format enclosed at Annexure – VII. More information on commercialization can be found at the website www.ebc.nic.in.

17. The Institute may not entrust the implementation of the work for which the grant is being sanctioned to another institution and to divert the grant receipts as assistance to the latter institution. However, in such situations the express permission of DBT may
be obtained. In case the grantee is not in a position to execute or complete the project, it may be required to refund forthwith to the Govt. of India (Department of Biotechnology) the entire amount of grant received by it.

18. The human resources that may be engaged for the project by the Institute are not to be treated as employees of the Govt. of India and the deployment of such human resource at the time of completion or termination of project will not be the concern/responsibility of the Govt. of India. The Organisation may make reservations for Scheduled Castes, Schedule Tribes etc. in the human resource to be engaged for the project in accordance with the instruction issued by the Govt. of India from time to time.

19. The Deptt. of Biotechnology reserves the right to terminate the grant at any stage and also to recover the amounts already paid if it is convinced that the grant has not been properly utilized or the work on the project has been suspended for any unduly long period or appropriate progress is not being made.

20. The project will become operative with effect from the date of release of the first installment for the project.

21. If the Investigator to whom a grant for a project has been sanctioned leaves the institution where the project is being implemented, he shall submit five copies of complete and detailed report of the work done by him on the project and the money spent till the date of his/her release and shall also arrange to refund the unspent balance, if any.

22. The organisation should maintain subsidiary accounts of the Govt. of India grant and furnish it to the Audit Officer as and when the recurring and non-recurring expenditure exceeds the limits of Rs. 5.00 lakhs.

Signature of Executive Authority of Institute with seal

Date : 27th December, 2010

Principal

Signature of Principal Investigator:
Name: Dr. Manju M. George

Date : 27th December, 2010

Office Seal