UNIVERSITI PUTRA MALAYSIA

THE CLONING, CHARACTERISATION AND ANTISENSE CASSETTES CONSTRUCTION FOR SOME PIGMENTATION GENES FROM ONCIDIUM SPP.

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By

SUGUMARAN MANICKAM

Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of Science in the Faculty of Food Science and Biotechnology Universiti Putra Malaysia

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DEDICATED TO MY BELOVED BROTHER WHO PASSED AWAY TRAGICALLY WHILE THIS RESEARCH WAS DONE

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science.

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Chairman: **Associate Professor Harikrishna Kulaveerasingam, Ph.D.**

Faculty: **Food Science and Biotechnology**

Genes that are responsible for color formation within the anthocyanin and carotenoid biosynthesis pathway have been isolated, studied and manipulated to produce novel colors in many plant species. Therefore, in this study, the genes that are involved in flower color formation namely CHS, F3H, DFR and PSY will be isolated and studied in the orchid species, *Oncidium goldiana*. The construction of antisense cassettes will also be carried out for CHS, DFR and PSY.

RNA was isolated from various stages of *Oncidium goldiana* and its purity and concentration was determined. Partial genes of CHS (605bp), F3H (503bp), DFR (418bp) and PSY (543bp) was isolated from flower petals by RT-PCR using degenerate primers. A BLAST search for homology revealed that these sequences had high homology at both the amino and nucleic acid levels with other known plant sequences.

The temporal and spatial gene expression studies using semi-quantitative RT-PCR showed that F3H and DFR were floral specific and they had the highest expression at the yellow buds stage. The level of CHS was the highest in partially opened flowers and PSY was expressed at its highest levels in green buds. CHS and PSY were not floral specific as they were also present in leaves.

The antisense cassettes were constructed for CHS, DFR and PSY. These vectors were driven by a 35S promoter and Nos 3' terminator sequence. The CHS vector was 13480bp, the DFR was 13380bp and PSY was 13430bp. These antisense cassettes were suitable for transformation by biolistic gun bombardment or via *Agrobacterium* infection.

Abstrak tesis yang dikemukakan kepeda Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

PENGKLONAN, PENCIRIAN DAN PEMBINAAN KASET "ANTISENSE" UNTUK BEBERAPA GEN PIGMEN DALAM *ONCIDIUM SPP.*

Oleh

SUGUMARAN MANICKAM

Jun 2000

Pengerusi : Prof Madya Harikrishna Kulaveerasingam, Ph.D.

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Gen-gen yang bertanggung jawab dalam pembentukkan warna sepanjang jalan "anthocyanin" dan "carotenoid" telah diasingkan, dikaji dan dimanipulasi untuk menghasilkan warna-warna baru ke atas pelbagai spesis-spesis tumbuhan. Oleh itu, dalam kajian ini gen-gen yang terlibat dalam pembentukkan warna bunga akan diasingkan dan diselidik dalam spesis orkid, *Oncidium goldiana*. Kaset-kaset "antisense" akan dibina untuk gen-gen CHS, DFR dan PSY.

RNA telah diestrakkan daripada pelbagai peringkat pertumbuhan *O.goldiana* dan ketulenan dan kepekatannya telah ditetapkan. Gen-gen separa CHS (605bp), F3H (503bp), DFR (418bp) dan PSY (540bp) telah diasingkan daripada kelopak bunga dengan kaedah RT-PCR menggunakan primer jenis "degenerate". Kajian BLAST untuk homologi menunjukkan bahawa jujukan gen-gen ini mempunyai homologi

yang tinggi terhadap jujukan asid amino dan asid nukleik daripada spesis-spesis tumbuhan yang lain.

Kajian ekspresi untuk berbagai peringkat pertumbuhan *O.goldiana* dengan menggunakan RT-PCR separuh-kuantiti menunjukkan bahawa F3H dan DFR adalah spesifik kepada bunga dan ia menunjukkan ekspresi yang paling tinggi pada peringkat kuntum-kuntum kuning. Penggumpulan gen CHS menunjukkan tahap ekspresi paling tinggi dalam bunga yang separuh buka manakala gen PSY menunjukkan ekspresi paling tinggi dalam kuntum hijau. Gen-gen CHS dan PSY adalah tidak spesifik kepada bunga kerana ia menujukkan ekspresi dalam daun.

Kaset-kaset "antisense" juga berjaya dibina untuk CHS, DFR dan PSY. Vektor-vektor ini mengandungi "promoter" 35S dan "terminator" Nos 3'. Vektor CHS ini bersaiz 13480 bp, DFR bersaiz 13380 bp dan PSY bersaiz 13430 bp. Kaset-kaset "antisense" ini sesuai ditransformasikan dengan menggunakan kaedah senapang biolistik atau dengan mikro-organisma.

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LIST OF ABBREVIATIONS

Symbol	Description
%	percentage
µg	microgram
µl	microlitre
°C	degree Centigrade
Amp	Ampicillin
bp	kilobase-pair
BSA	Bovine Serum Albumin
CaMV	Cauliflower Mosaic Virus
cDNA	Copy Deoxyribonucleic Acid
CHS	Chalcone Synthase
cm	centimeter
Da	Dalton
dATP	2' - Deoxy-adenosine-5' - triphosphate
dCTP	2' - Deoxy-cytidine-5' - -triphosphate
DEPC	Diethyl Pyrocarbonate
DFR	Dihydroflavonol 4-Reductase
dH_20	sterile distilled water
DNA	Deoxyribonucleic Acid
dGTP	2' - Deoxy-guanosine -5' - -triphosphate
dTTP	Thymidine -5' - -triphosphate
EDTA	Ethylenediaminetetraacetic Acid

EtBr	Ethidium Bromide
F buffer	Formaldehyde buffer
F3H	Flavanone 3-hydroxylase
g	gram
GTE	Glucose- tris-EDTA
GUS	β-glucuronidase
hr	hour
kb	kilobase-pair
LB	Luria-Bertani
M	Molar
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MMuLV	Moloney Murine Leukaemia Virus
MOPS	3-[N-Morpholino] propanesulfonic acid)
MW	molecular weight
ng	nanogram
O.D.	Optical Density
ori	origin of replication
PSY	Phytoene Synthase
rpm	revolution per minute
SAPH	ρ-Aminosalicylic Acid
SDS	Sodium Dodecyl Sulphate

TAE	Tris Acetate EDTA
Taq	*Thermophilus aquaticus*
TNS	Tri-isopropylnaphtalene (Sodium salt)
Tris	Tri-isopropylnaphtalenesulfonic Acid

INTRODUCTION

Introduction

Flower breeding dates back several centuries and in the beginning has been more of an art than a science. In the past century, flower breeding has grown in popularity, especially in the Netherlands, with the result that vast improvements have been made in phenotypic and production characteristics of many ornamental plants. The creation of new color patterns in ornamentals has always been accompanied by a detailed chemical and biochemical analysis of the components responsible for color formation (Akavia *et al.*, 1981; Griesebach, 1983; Harbone, 1975; Vidal *et al.*, 1977).

Recently, gene isolation, manipulation and transfer technologies in combination with improved knowledge of pigment and hormone biosynthetic pathways have opened up novel routes to the development of improved ornamental plant varieties. *Petunia hybrida* has been one of the major subject of investigation and today 32 genes

have been described which affect flower pigmentation in Petunia (Wiering *et al.*, 1979).

Objective

The objective of this research is to isolate and characterize the genes that control the main enzymes that are involved in the flower color production pathway of *Oncidium spp*. Therefore 3 genes from the flavonoid biosynthetic pathway and 1 gene from the carotenoid biosynthetic pathway have been identified. These important enzymes which are targeted for study are:

1. Chalcone synthase (CHS)
2. Flavanone 3-hydroxylase (F3H)
3. Dihydroflavonol 4-reductase (DFR)
4. Phytoene synthase (PSY)

In addition to this, antisense transformation vectors driven by the S35 promoter will be constructed for:

1. Chalcone synthase (CHS)
2. Dihydroflanonol 4-Reductase (DFR)
3. Phytoene synthase (PSY)

LITERATURE REVIEW

Flower Color

Flower color is a key element in consumer selection between ornamental varieties available in the market place. For some ornamental species, however, only a narrow color spectrum is available, while in others specific colors, like blue, are lacking. With quite detailed knowledge of how anthocyanins and other pigments are made in flowers, and with some sketchy ideas of how the amount and distribution of anthocyanin production is controlled, the possibility of genetic engineering of flower color has become a reality (Meyer *et al.*, 1987; van de Krol *et al.*, 1988; van der Krol *et al.*, 1990 Napoli *et al.*, 1990). Some of the results obtained in these experiments have been unexpected (van der Krol *et al.*, 1990; Napoli *et al.*, 1990) and may lead to a greater understanding of the systems involved in controlling floral pigmentation. New colours may be provided by the transformation of genes from species which can catalyse a particular anthocyanin modification into those that cannot. Pale varieties may be produced by inhibition of gene action through antisense technology. Unexpectedly, it seems that new pigmentation patterns may also arise with this approach (van der Krol *et al.*, 1988). Thus, the introduction of new color and forms through genetic engineering is likely to have a large influence on the industry and this research area has attracted the attention of several biotechnology companies.