UNIVERSITI PUTRA MALAYSIA

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

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

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

June 2000

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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June 2000

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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I certify that the examination Committee met on 16th June 2000 to conduct the final examination of Sugumaran Manickam on his Master of Science thesis entitled "The Cloning, Characterization and Antisense Cassette Construction of some Floral Pigmentation Genes in *Oncidium Spp.*" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

Signed

Sugumaran Manickam

Date: 30/6/2000

TABLE OF CONTENTS

		Page
DEDICATION		ii
ABSTRACT		iii
ABSTRAK		v
ACKNOWLEDGEMENTS		vii
APPROVAL SHEETS		ix
DECLARATION FORM		xi
LIST OF TABLES		xv
LIST OF FIGURES		xvi
LIST OF PLATES		xvii
LIST OF ABBREVIATIONS/NOTATIONS/GLOSSARY OF TERMS		xix
CHAPTER		
I	INTRODUCTION	1
	Objective	2
II	LITERATURE REVIEW	3
	Flower Color	3
	The Flower Industry	4
	The Family Orchidaceae	5
	The *Oncidium*	6
	Anthocyanin Biosynthetic Pathway	9
	Chalcone Synthase (CHS)	11
	Flavanone 3-Hydroxylase (F3H)	13
	Dihydroflavonol 4-Reductase (DFR)	14
	The Distribution and The Function of Carotenoids	17
	The Carotenoid Biosynthetic Pathway	19
	Antisense vs Sense Genes	22
III	MATERIALS	26
	Plant Materials	26
	Primers, Clones and Plasmids	26
IV	TOTAL RNA EXTRACTION FROM TISSUES OF *ONCIDIUM GOLDIANA*	28
	Introduction	28
	Methods	29
	RNA Extraction	29
	RNA Agarose-Formaldehyde Gel Electrophoresis	31
	Results and Discussions	32
	RNA Extraction	32
	Quantification of Total RNA by Spectrophotometry	33

V CLONING OF CHS, F3H, DFR, PSY RT-PCR AMPLIFIED PRODUCT 38
Introduction 38
Materials and Methods 39
Reverse Transcription (RT) 39
Polymerase Chain Reaction (PCR) 40
TOPO TA Cloning of PCR Products 41
Plasmid Miniprep 42
Sequence Analysis 43
Results and Discussions 44

VI GENE EXPRESSION STUDIES USING SEMI QUANTITATIVE-RTPCR (SQ-RTPCR) FOR CHS, F3H, DFR AND PSY 49
Introduction 49
Materials and Methods 50
Results and Discussions 52

VII THE CONSTRUCTION OF ANTISENSE TRANSFORMATION CASSETTES 58
Introduction 58
Materials and Methods 59
Plasmid pHK17 59
Preparing pHK17 for Cloning 59
Preparing the Insert for Cloning 60
Ligation and Transformation of pHK17 and Insert 61
Double Digestion of pHK17 cloned with partial CHS, DFR and PSY Inserts 61
Double Digestion of pCAMBIA 1301 62
Ligation of Purified pCAMBIA 1301 and pHK17 Cloned With CHS, DFR and PSY 65
Results and Discussions 65

VIII CONCLUSION 71

VII BIBLIOGRAPHY 73

APPENDICES 81
APPENDIX A 82
APPENDIX B 83
APPENDIX C 84
APPENDIX D 85
APPENDIX E 86
APPENDIX F 87
APPENDIX G 88

BIODATA OF AUTHOR 89

LIST OF TABLES

Table		Page
1	Spectrophotometric Readings of RNA Isolated From Various Tissue Samples..	36
2	Absorbency Ratios and Total RNA Yield From Various Tissues..	36
3	Various PCR Buffers Used for PCR and its compositions	41
4	PCR Conditions for CHS, F3H, DFR and PSY...........................	44

LIST OF FIGURES

Figures		Page
1	Anthocyanin and Flavanol Biosynthetic Pathway......................	10
2	General Structure of the Flavonoid Skeleton.........................	12
3	Carotenoid Biosynthesis in Plants......................................	20
4	Partial Sequence of CHS, F3H, DFR and PSY.......................	48
5	Expression Levels of CHS..	56
6	Expression Levels of F3H..	56
7	Expression Levels of DFR..	57
8	Expression Levels of PSY..	57
9	Plasmid pHK 17..	63
10	Plasmid pCAMBIA 1301..	64
11	35S Driven Antisense Expression Cassettes for Chalcone Synthase (CHS)..	68
12	35S Driven Antisense Expression Cassettes for Dihydroflavonol 4-Reductase (DFR)..	69
13	35S Driven Antisense Expression Cassettes for Phytoene Synthase (PSY)..	70

LIST OF PLATES

Plate		Page
1	*Oncidium goldiana*..	8
2	Stages of *O.goldiana*..	27
3	Open Flowers of *Oncidium*..	27
4	Total RNA from Green Buds..	35
5	Total RNA from Yellow Buds..	35
6	Total RNA from POF..	35
7	Total RNA from OF..	35
8	Total RNA from Leaves..	35
9	CHS, F3H and DFR bands after RT-PCR	47
10	PSY band after RT-PCR..	47
10	Expression Levels of CHS..	54
11	Expression Levels of F3H..	54
12	Expression Levels of DFR..	55
.13	Expression Levels of PSY..	55
14	pCAMBIA 1301 Digested With Pst1 and Sma1	66
15	CHS (Antisense) Cloned in pCAMBIA1301 and Digested With Pst1 and Sma1..	66
16	DFR (Antisense) Cloned in pCAMBIA1301 and Digested With Pst1 and Sma1..	66
17	PSY (Antisense) Cloned in pCAMBIA1301 and Digested With Pst1 and Sma1..	66
18	PCR CHS Antisense..	67

19	PCR DFR Antisense	67
20	PCR PSY Antisense	67

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.