BARD

FINAL REPORT
PROJECT NO. IS-2192-92

**Molecular Analysis of Carotenoid Biosynthesis in
Plants: Characterizing the Genes *Psy*, *Pds* and
*CrtL-e***

J. Hirschberg, G.A. Moore

1997

Bard Project No. IS-2192-92R

Title:

**MOLECULAR ANALYSIS OF CAROTENOID
BIOSYNTHESIS IN PLANTS: CHARACTERIZING THE
GENES , *Psy*, *Pds* and *CrtL-e***

JOSEPH HIRSCHBERG
Department of Genetics
The Hebrew University of Jerusalem
Jerusalem, 91904 Israel
Tel. (972) 2-6585662;
FAX: (972) 2-5633066
E-mail: HIRSCHU@vms.huji.ac.il

GLORIA A. MOORE
Horticultural Sciences Department
Plant Cellular and Molecular Biology
Program, Institute of Food and Agricultural
Sciences
University of Florida
Gainesville, FL 32611
U.S.A.
Tel. 904-392-7593
FAX: 904-392-5653

Project's starting date: 8/1993

Final Scientific Report*

Signature

* Following the instructions of the director of BARD (letter dated 8 December 1996) this report includes only the part of the Israeli PI.

TABLE OF CONTENTS

ABSTRACT	3
DESCRIPTION OF THE RESEARCH ACTIVITIES AND RESULTS	4
OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL	4
RESULTS	5
Expression of <i>Pds</i> during fruit ripening	5
Promoter analysis of <i>Pds</i>	7
Over-expression of <i>Pds</i> and antisense of <i>PDS</i> in transgenic plants	10
Genetic engineering of herbicide resistance	14
Cloning the cDNA of lycopene ϵ -cyclase	17
Sequence analysis, phylogeny and evolution of the lycopene cyclase genes ..	20
Expression of <i>CrtL-b CrtL-e</i> during tomato fruit ripening	23
Expression of <i>Pds and CrtL-e</i> during fruit ripening in the color mutant <i>Del</i>	25
Immunological detection of phytoene desaturase	26
METHODS	28
Cloning and sequence analysis	28
Pigment extraction and analysis	28
Extraction of RNA	29
Measurement of mRNA by RT-PCR	29
Immunological protocols	30
CONCLUSIONS	31
REFERENCES	31
DESCRIPTION OF COOPERATION	32
EVALUATION OF THE RESEARCH ACHIEVEMENTS WITH RESPECT TO THE ORIGINAL RESEARCH PROPOSAL AND ITS OBJECTIVES	32
PUBLICATIONS	33
APPENDICES	35

ABSTRACT

In this research we have studied the molecular biology of carotenoid biosynthesis in tomato. The investigations focused on the genes *Pds* and *Psy*, encoding phytoene desaturase and phytoene synthase, respectively, which are key enzymes in the biosynthetic pathway of lycopene and β -carotene. In addition, we have investigated the genes for lycopene cyclase. We have cloned from tomato and characterized the cDNA of *CrtL-e*, which encodes the lycopene ϵ -cyclase, and analyzed its expression during fruit development. The results establish a paradigm for the regulation of carotenoid pigment biosynthesis during the ripening process of fruits. It is concluded that transcriptional regulation of genes that encode carotenoid-biosynthesis enzymes is the major mechanism that governs specific pigment accumulation. During the ripening of tomato fruits, transcription of the genes encoding the enzymes phytoene synthase and phytoene desaturase is up-regulated, while the transcription of the genes for both lycopene cyclases decreases and thus the conversion of lycopene to subsequent carotenoids is inhibited. These findings support the working hypothesis of the molecular approach to manipulating carotenogenesis by altering gene expression in transgenic plants, and offer obvious strategies to future application in agriculture. The molecular and physiological knowledge on carotenogenesis gained in this project, suggest a concept for manipulating gene expression that will alter carotenoid composition in fruits and flowers.

DETAILED DESCRIPTION OF THE RESEARCH ACTIVITIES AND RESULTS

INTRODUCTION

The overall goal of this research was to study carotenoid biosynthesis at the molecular level in order to eventually be able to manipulate fruit color (and carotenoid composition) through genetic transformation techniques utilizing the cloned carotenoid biosynthesis genes - *Pds* and *Psy*. These genes encode phytoene desaturase and phytoene synthase, respectively, which are two key enzymes for the early steps in the biosynthetic pathway of lycopene and β -carotene.

Specific goals of the research were to study the regulation of expression of *Pds* and its influence on carotenoid biosynthesis and explore the possibility for enhancing fruit colors by over-expressing *Pds* and *Psy* in transgenic plants. Additional goal was to produce resistance to "bleaching herbicides" in transgenic plants that contain a genetically modified *Pds*. This trait is potentially useful as a selectable marker for gene transformation in plants.

Parts of the research on *Pds* and *Psy* was conducted in tobacco and tomato plants. Tobacco is easier to manipulate by gene transformation and, therefore, was used as the first choice for producing transgenic plants. However, transformation of tomato has also been carried out.

During the first year of the research it has become apparent that parts of the research plan that relate to *Psy* were being carried out by another group in the UK. Therefore, we have added new goals to the original proposal, however, on the same line of investigation. Accordingly, we have studied the genes for lycopene cyclases in tomato - *CrtL-b*, which encodes the lycopene β -cyclase and *CrtL-e*, which encodes lycopene ϵ -cyclase. The cDNA of *CrtL-b* has been cloned and molecularly characterized in our laboratory in the framework of a separate program. In this program we have cloned and characterized the cDNA of *CrtL-e*. In addition, we analyzed the expression of both genes during tomato fruit ripening.

The major and most significant outcome of this research is the explanation for the accumulation of lycopene in tomato fruits ("why tomatoes are red"). Our results indicate that transcriptional regulation of carotenoid biosynthesis genes is the key mechanism for over-production and accumulation of lycopene. During the ripening process the transcription of

genes encoding the enzymes phytoene synthase and phytoene desaturase is up-regulated, while the transcription of the genes for both lycopene cyclases decrease and thus the conversion of lycopene to subsequent carotenoids is inhibited.

RESULTS

Expression of *Pds* during fruit ripening

Tomato fruit develop over a period of about seven weeks and ripen in five to ten days. This process has been subdivided into stages for convenience and consistency in characterization [1,2]. The first phase in which external color is apparent is referred to as the "breaker" stage. Subsequent stages are characterized by some researchers according to the color of the fruit as it ripens, e.g. yellow, orange, red.

Previously we have shown that transcription of *Pds* is up-regulated during fruit ripening in tomato. To determine more accurately the increase in the abundance of transcripts of *Pds*, total RNA was extracted from various stages of fruit ripening and poly(A)⁺ mRNA was purified. This enrichment step for mRNA was necessary since preliminary data indicated that steady state levels of *Pds* are very low. Northern blot analysis was carried out using the cloned cDNA of tomato *Pds* as a molecular probe. The results (shown in Fig. 1) demonstrate that *Pds* mRNA increases at the "breaker" stage, peaks at "orange" and decreases a little in "red". Overall increase is estimated to be 10 fold relative to the mature green stage.

In later stages of the investigation we developed a better method to determine the amount of mRNA of carotenoid genes, which is based on reverse transcription plus polymerase chain replication (RT-PCR) [3]. The results of mRNA measurements with this new technique confirmed the data presented in Fig.1. Similar results have been obtained for *Psy* [4,5].

Figure 1: Measurement of mRNA of *Pds* during fruit ripening. Northern blot analysis (A) was carried out with RNA extracted from various stages of ripening. RNA was separated by gel electrophoresis. Each lane was loaded with 3 μ g of poly A⁺ RNA. The RNA blot was probed with ³²P labeled *Pds* cDNA. Relative radioactivity in each band was determined by phospho-imager and densitometry of the autoradiogram and is presented in the histogram (B).

Promoter analysis of *Pds*

The up-regulation of *Pds* expression during fruit ripening, as manifested by an increase in the steady-state levels of its mRNA, could have resulted from differential transcription or from other, post-transcriptional processes. To determine the actual mechanism we have analyzed the promoter of *Pds* since “run-on” transcription experiments are futile in the case of carotenoid genes, whose transcription is extremely low.

For that purpose we cloned the genomic sequence of *Pds* from tomato and determined its nucleotide sequence (Fig. 2). A region of 565 bp upstream to the transcription initiation site was fused with the promoter-less coding region for the β -glucuronidase (GUS) gene (Fig. 3). Two deletions: d224 and d306, which maintain 341 and 159 bp, respectively, of the sequence upstream to the initiation site were also fused with GUS. These constructs were subcloned in Ti vector pBIB101. Transgenic tobacco plants have been generated with these constructs and expression of GUS was assayed fluorimetrically in tissue extracts of leaves and flowers (Fig. 3).

The results indicated that the up-regulation of GUS activity in flower tissues was due to increased transcription from the *Pds* promoter, which was induced in a heterologous system of transgenic tobacco. Similar results have been obtained recently in an analogous experiment in tomato [6]. The significance of these results is that **regulation of transcription** is most probably the primary mechanism of regulation of phytoene desaturase in chromoplast-containing tissues (fruits and flowers).

1 AAGCTGACGATTTTCCTTCAGAGTAGAAATTGAAAAGAATCAACTAAAAAGGATAGTCCTTCGATTGATTTCCGGCTTAAAAATAAACTAATAAGAATGAGAG
106 AGCGAATAATAGAAATATTTTGAAATTTTAAAGATATTTCAACTATGTAAATTGCGTTATAAATTTCTTAAATTAGTAGCACCTAATAGTTTAGTCTCAAAAGTC
211 AAAACTACTACATAATCGTGCTCATTTTTCACATTAAATGCCTACATGATGTAAAGTAAAACTCGTAGCATTCTACGTGTTTTACTCAACTCAAACATCCTGT
316 TCATTTTAATAAACGTACGATGAGCTTCTCTCTCCAAATTTCTTTTCTTTTAAAAAATATTTTTTTTATATCAATCCAAATGGGCTCCAAATTTAT
421 CATAAATTAGGTAGAACTTAGATATTAAGAAAAGAAAGGGTTTATCTCGCAAGTGTGGCTATGGTGGGACGTGTCAAATTTGGATTGTAGCCAAACATGAGA
526 TTTGATTAAAGGGAATTGGCCAAATCACCAGAAAGCAGGCATCTTCATCATAAATAGTTTGTATTATTTATACAGAATTATACGCTTTTACTAGTTATAGCATT
631 GGTATCTTTTCTGGGTAACGTGCCAAACCACCACAAATTTCAAGTTTCCATTAACTCTTCAACTTCAACCCAAACCAATTTATTGCTTAATTGTGCAGAACCA
736 CTCCCTATATCTTCTAGGTGCTTTCATTGCTTCCGAGGtaagaaaagattttgtttctttgaatgctttatgccactcgtttaactctgaggtttgtggtatct
841 tttaggcgactttttttttttgtatgtaaaattgtttcataaatgcttctcaacataaatcttgacaagagaaggaattttaccaagattttaggttcaga
946 aatggataattttcttactgtgaaatatccttatggcagggttttactgttatttttcagTAAATGCCTCAAATTGGACTTGTTCCTGCTGTTAACTTGAGAGTC
1 M P Q I G L V S A V N L R V
1051 CAAGGTAGTTCAGCTTATCTTTGGAGCTCGAGGTCGTCTTCTTTGGCAACTGAAAGTCGAGATGGTTGCTTGCAAAGGAATTCGTTATGTTTGTGCTAGCGAA
15 Q G S S A Y L W S S R S S S L G T E S R D G C L Q R N S L C F A G S E
1156 TCAATGGGTCATAAGTTAAAGATTTCGTACTCCCATGCCACGACCAGAAGATTGGTTAAGGACTTGGGGCCTTTAAAGgtttggtttgaattctatagactgat
50 S M G H K L K I R T P H A T T R R L V K D L G P L K
1261 acttgaattattatctcaaggcatatatttctctaaaaataaaggaactatcttgcattcagGTCGTATGCATTGATTATCCAAGACCAGAGCTGGACAATACAG
76 V V C I D Y P R P E L D N T V
1366 TTAATCTTTGGAGGCTGCATTTTTATCATCAACGTTCCGTGCTTCTCCGGGCCCACTAAACCATTGGAGATTGTTATTGCTGGTGACGgtgatattccgggtc
91 N Y L E A A F L S S T F R A S P R P T K P L E I V I A G A G
1471 ATCTATATTTGTAGCATTCGTTTTGCTTATTTcgaaggaagcattttttgtctgtattatcatttgaatattttattgtacctgtacatgcctgattaactgtttg
1576 actacaaaatttgaggttattaaagcctctgttcatttttgcgactaaatagaaagtcactgctgattggactaggagcagttacacaagttgtaaattttggacta
1681 ttcagccatcactctctttatttctgctgttataatgtcttctctctatcatttgagccatgataagtcactgaacatgctattgtctgtgacatgtccaaat
1786 ggtgactctgaagtattagaggttgtatataatctccagtgcaagattgatccatccactatacctatatattgacagtattgtggagttccaaaatttga
1891 acactaaattaaagtacacaataaaggggttgcaacctttaatgcatgcaatctgtgagaacgtgaagcagatatatgatagatcttcatatttcatcttactat
1996 gctggagcagtgaccttaagctgccttgaactgttcattgacttgggtgcaaaatactgtaacattcattttcaactagtactataatcaactagtagaaaat
2101 tataactacctaagaagtggtatttgcagatttgttccattgtacttcaaaagcatataataaagaaggcaacgcaaggtactagatagctatttcagataa
2206 atggagcagtaacatgattctatgataattcaacttttggaaatatcagGTTTGGGTGGTTTGTCTACAGCAAAATATTGGCAGATGCTGGTCACAAACCGATA
121 L G G L S T A K Y L A D A G H K P I
2311 CTGCTGGAGGCAAGGGATGTTCTAGGTGCAAGgttaagaagatcaaaaaattccttttttttaaaaaatcctttttctttttgtgtctttccctatttagtagtc
139 L L E A R D V L G G K
2416 ctttttggggtaggggttctgttttgcataataatgtgctgtctttctggttagattctacgtaacctagcctaataaccttttagtctagatcactatgttaagta
2521 gtttcaaaaacaaaaactctctcttagtttgttttatgttttgatgcattgtttctctaaagattagatgtcctttttgtctacttgcttttgttacatttgcatt
2626 ttagggtgcaagttggtatttgcatacttaataatgcatgttttaattttacttttctgctattctatgtcctttaatcaagaatcgatttgcacgtatttctt
2731 cctcgtattttatgcattctatatgtcttcaagttgaaatggctagaatggattatcttgcctattggagtcaccaagaattctagttgtataggcaaaacattgat
2836 ttagtattagctcttattgtgttctactgttgagaatttatatctcccttagGTAGCTGCATGGAAAGATGATGATGGAGATTGGTACGAGACTGGTTTGCATAT
150 V A A W K D D D G D W Y E T G L H I
2941 ATTCTgtaagtttgacctctcattgttatatgtttacgttaattcttctatatactgtcattgtatttttttttttgatctctagccaatttagacatctcctatcc
168 F F
3046 tctgtttgtcgtttatcgtttatcttttcaaaaaatagcctattattgtcagtaaaatctgtattttgtctagcttctcctttctcatcttattattcatatagta
3151 ctcatacaaaattggtgcttgcattctcttttaagTTGGGGCTTACCCAAATATTGAGAATTTAGGGATTAAACGATCGATTGCAATGGAAGGAACA
170 G A Y P N I Q N L F G E L G I N D R L Q W K E H
3256 TTCAATGATATTTGCAATGCCAAGCAAGCCAGGAGAATTACGCGCTTTGATTCTCCGAAGCTTTACCCGCTCCTTTAAATGgtgagcctaatacagagtaaaatt
194 S M I F A M P S K P G E F S R F D F S E A L P A P L N G
3361 tctcctctttagtatttttgttaaacttccctaataagctgtaaagttgattagaattctaaaaaaaatctgtaaaattgataagtcacacacattatgg
3466 gactttactaaccttaaaagagcataaaagttcattacttcttctcattggaccttttgtgtgcagctaaaaatattaaattctttgatataaatttgcagGAATTTTA
222 I L
3571 GCCATCTTAAAGAATAACGAAATGCTTACATGGCCAGAGAAAGTCAAATTTGCAATTGGACTCTTGCCAGCAATGCTTGGAGGGCAATCTTATGTTGAAGCTCAA
224 A I L K N N E M L T W P E K V K F A I G L L P A M L G G Q S Y V E A Q
3676 GATGGGATAAGTGTAAAGGACTGGATGAGAAAGCAAGtgctgaatcaattatgttgctttttaaagtcataaaacctggctatatagttaccagttcacacacaa
259 D G I S V K D W M R K Q G
3781 attttcatttgaggtgttagattgtctactgcctctcttttgcataagagcatacatctagcaaaatctcagcagcagcaagttgctttgcaggGTGTGCCGGACA
272 V P D R
3886 GGGTGACAGATGAGGTGTTTATTGCTATGTCAAAGGCACTCAACTTTATAAACCTGACGAACTTTCAATGCAGTGCATTTTATGATCGCATTGAACAGGTTTCTTC
276 V T D E V F I A M S K A L N F I N P D E L S M Q C I L I A L N R F L Q

3991 AGgttagaatcctgatccacattcaaaacaaagagagagagagaagtacttttgccttccagactctcacataacatgaaatctctaactaaggagcttaaa
 4096 tgtgattgggtgtgccataagttcatcttgtttgttacgcaacacttagattgctgtaggaaacattcataatggcagttttgaagatgaaacttttgaagttacc
 4201 atccgtcaaaataataactttgttttgcctgttacttctggaggacaggtttctctaggttgagggcatattgtttaactgctggagaagtttttgtttaatc
 4306 gacatactctcagaagcttctactagcagaaatggaactgatttatgctaacttggacacttatattaaccatcctctcctcttttcttttccattactttt
 4411 ctgaatcatcttcaggaaacttctattaaacttctccaaattctaccatgcaaatggcctcttgtaaattataccctgaatctcatggatgaaggactactttac
 4516 tgattcacttttaaacagctcagttgtcatctagtagcattttattgtcttaagaaatttaagggtgaactacttataacttggaaacaaacacccgaaaga
 4621 ataagaaatgatggtgaatttctccattcagtttagcttttctgatataattgattataattaacagGAGAAACATGGTTCAAAAATGGCCTTTTAGATGGTAAT
 311 E K H G S K M A F L D G N
 4726 CCTCCTGAGAGACTTTGCATGCCGATTGTTGAACACATTGAGTCAAAAGGTGGCCAAGTCAGACTGAACTCACGAATAAAAAAGATTGAGCTGAATGAGGATGGA
 324 P P E R L C M P I V E H I E S K G G Q V R L N S R I K K I E L N E D G
 4831 AGTGTCAAGAGTTTATACTGAGTGACGGTAGTGCAATCGAGGGAGATGCTTTTGTGTTTCCGCTCCAGgtataatatccattatactagttgattaatccagt
 359 S V K S F I L S D G S A I E G D A F V F A A P V
 4936 tttcagtttcttaatatgagttatgatttttgcctgatttttgatgaaccaattagTGGATATTTTCAAGCTTCTATTGCCTGAAGACTGGAAAGAGATTCCATAT
 383 D I F K L L L P E D W K E I P Y
 5041 TTCCAAAAGTTGGAGAAGTTAGTCGGAGTACCTGTGATAAATGTACATATATGgttagtggtgaatataattttacttgttagtggtttgagagatgcatacctatg
 399 F Q K L E K L V G V P V I N V H I W
 5146 tatgtgcaatgaaatgtctatacatgtatgtttataaatattgacttgcatactcctcagtccttgtgtgaatttcccttgatgaggaattatggatgttgggttt
 5251 tccgaaacttagatagtaaaagtgcgaatttctgtacataatttaagtagaagaactaccatcgcttctggccattagtggctaataattctgctgaaaagaaaaaa
 5356 attctcatcatgaattcatatgtatccttaataatactgaagcgactacagttatcggtaccaaaccaataacgattgtataaattaaactgcGTTTGACAGAAA
 417 F D R K
 5461 ACTGAAGAACACATATGATCATTGCTCTTCAGCAGgttaatttcattcactttattcttccaaagcagtttgtgtgcgatgacttcattttcatttagttccc
 421 L K N T Y D H L L F S R
 5566 ttcctcctcttccagtgctcattattttgatacaacattgagtagacggattatcaatttccctgcaaatttccctagtgaacttaaatcattagattcatatcatt
 5671 gtaaatggctatgttttaataagaagtttaagtaagtttttattggtctaaataagcatttgtgttggccctcccccctcccggtgaaatccttctctaagcatt
 5776 agctggtgagattcagttgaggatagacatgacatgtcagggcaaccacttctccgtggcatgaaaaatggactagagatocaagtcgtcacatttcatcagcta
 5881 tatgttaattatgttccctttagtttttaaatgacaccggctggtggagacagataaataagagctccttttcaattcttaaaaaattgtgttgtttatgtac
 5986 ttcatttgggttccctttcttaatttctcttccatattcatgcagAAGCTCACTGCTCAGTGTGTATGCTGACATGCTGTACATGTAAGgtattcatatgtaac
 433 S S L L S V Y A D M S V T C K
 6091 catttatattgtctcacaattataatctgtggacttgtgtataatttgcctgtgaattcgataatgtctgttggtaagtgtatgaattcccgctgttgttaattctca
 6196 tttggatatacaatgatattgttttcttatatatggaatagccagggctatgatcctgtctgtgcacggggcccgagccaccacatgaggcaagttacacaggac
 6301 aagtaagaaattttgacgaaacttagccggagatttttttttccattttagctaattttctaaagaatttaattgggataaaaattcttttattagctatctaggt
 6406 tcttgccttctgggggtggaggggttaagaagagaacttaattcagtggttacctgcagtttggaaagagctctacagatctacttctcagttttattgtgtgtgg
 6511 aaatctgtacaaatataaagcatttgcacaaaagtgtgagaagtttaacttgaaaaaagaggttatgtatagaacactcagacaattgtctatcgtaaaaaatttt
 6616 tcccttgcctgtgtatagtcattatccattctcttcatgtgtgttttacgacctctgacatagctggcctctatgcagGAATATTACAACCCCAATCAGTCTAT
 448 E Y Y N P N Q S M
 6721 GTTGGAAATTGGTTTTTGCACCTGCAGAAGAGTGGATATCTCGCAGCGACTCAGAAATTATTGATGCAACGATGAAGGAAGTAGCAACGCTTTTTCTGATGAAAT
 457 L E L V F A P A E E W I S R S D S E I I D A T M K E L A T L F P D E I
 6826 TTCAGCAGATCAAAGCAAAGCAAAAATATTGAAGTACCATTGTTGCTAAAACCTCCGAGgttaatatagcatttgccttcatagttgctcatcatgatatttttt
 492 S A D Q S K A K I L K Y H V V K T P R
 6931 cactcttcatacaaaatatttagatcatatttgagagtcctgcatttactagaatcgagtcgaatggagtcctatacatgaatccttgttgatctgcttttttct
 7036 ctcagGTCTGTTTATAAACTGTGCCAGGTTGTGAACCTGTCCGCCCTTTACAAAGATCCCAATGAGGGGTTTTATTATTAGCCGGTGACTACACGAAACAGAAA
 511 S V Y K T V P G C E P C R P L Q R S P I E G F Y L A G D Y T K Q K
 7141 TACTTGGCTTCAATGGAAGCGCTGTCTTATCAGGAAAGCTTTTGTGCTCAAGCTATTGTACAGgttaagttctcactggttgcagcagttgtttcttctcattcct
 544 Y L A S M E G A V L S G K L C A Q A I V Q
 7246 ggtatgttttgattatattctgtcttcttcttggtagagaagtgtagaaccatttcttaggcattacatttggtagcagtcctcttctgtgaagcctgtttctc
 7351 attaatgtggcgggagagaggggtgtcaagtagtacaaacttcaacttctgacggagcttcttctctcaacttgtacettcaacagaaatggatgtgcaacatgc
 7456 ctataacttctctgcctagctgaatagtttctccaaccaaatttaagatttctgacacacacaggttagtcaaggagtaactgtagtcctccattcttaattatgg
 7561 tcttgggtttgatcccttggaaaaggagaaaatgttggagggagcaatttccctttaaaccggcacycaaacagataaggttggacgagtgagtttgaataacttgg
 7666 tggattaacaacaaaaaattggacggctacagatgcagtggtgtgtgtcttagaacctcagtagtagcgtgataatgcagagagttgtgttattcatctttata
 7771 cacctatatttgcagGATTATGAGTTACTTGTGGACGTAGCCAAAAGAAGTTGTGCGGAAGCAAGCGTAGTTAGCTTTGTGGTTATTATTATTAGCTTCTGTACAC
 565 D Y E L L V G R S Q K K L S E A S V V *
 7876 TAAATTTATGATGCAAGAAGCGTTGTACACAACATATAGAAGAAGAGTGCAGGAGTGAAGCAAGTAGGAGAAATGTTAGGAAAGCTCCTATACAAAAGGATGGCAT
 7981 GTTGAAGATTAGCATCTTTTAAATCCCAAGTTTAAATATAAAGCATATTTTATGTACCACTTTCTTTATCTGGGGTTTGTAAATCCCTTTATATCTTTATGCAATC
 8086 TTTACGTTAGTTAATATCTATCTATCGATATTTCTAGTATCTTATACTATAGATCCAACGAACCAAGATTATGAACCGTGTCTTCCAGAAATCTAATAATGAT
 8191 GGGAGCAATATAAATATAAGGATGTCTTTGACAATAAAGGGCGGTGAAGAGTTATAGTGAAGCTT 8258

Figure 2: Nucleotide sequence and deduced amino acid sequence of the gene *Pds* from tomato. Introns are shown in low case letters. Predicted TATA and CAAT consensus sequences are underlined. Transcription starts at nucleotide 561, based on cDNA data.

GUS expression (relative units)

<i>PDS</i> promoter		leaves	sepals	petals	ovary
	565 bp	100	3,400	42,300	21,400
	341 bp	53	2,014	17,100	186,100
	159 bp	5			

Figure 3: Schematic structures of the *Pds* promoter-GUS fusions and GUS activity (in relative units) in different tissues of transgenic tobacco plants.

Over-expression of *Pds* and antisense of *PDS* in transgenic plants

Tomato (cv. VF36) and tobacco (SR1) plants were transformed with the full length cDNA of *Pds* from tomato fused to the strong constitutive promoter of the CaMV 35S in the binary Ti plasmid pBIB, either in the sense (pBOX) or antisense (pBAS) orientations (Fig. 4).

Transformation was done with *A. tumefaciens* strain LBA4404. In both the sense and antisense transformation a strong inhibition of carotenogenesis was observed at the callus stage of regeneration in tomato. The inhibition was at the phytoene desaturation. Few tomato plants which expressed antisense of *Pds* at a lower level were regenerated. These plants exhibited bleached phenotype and eventually died (fig. 5). The appearance of the dying plants resembled drought wilting. This can be due to deficiency in abscisic acid (ABA), which is synthesized from carotenoids (violaxanthin or neoxanthin).

Carotenoid analysis in leaves of the transgenic plants confirmed that the phenotype was indeed a result of inhibition in the leaves of carotenoid biosynthesis at the step of phytoene desaturase (Fig. 6).

Figure 4: Structure of plasmids pBOX and pBAS for expression of *Pds* in plants.

Figure 5: Transgenic tomato plants that were transformed with either the pBIB vector alone (= "WT" control) (left), or with the pBAS plasmid (antisense expression of *Pds*) (right).

Figure 6: Carotenoids composition in leaves of wild type and transgenic tomato plants that over express antisense of *Pds*. Carotenoids were extracted from leaves and analyzed by HPLC. Normally, phytoene never accumulates in leaves to any detectable level. The accumulation of phytoene in the transgenic plants indicate that the reduced carotenoid level was due to inhibition of phytoene desaturase, which caused buildup of the precursor.

A weaker phenotype of bleaching was observed in tobacco transformants. Five transgenic tobacco plants that over-expressed *Pds* were regenerated. The presence of 1-2 copies of transgenic *Pds* was confirmed by Southern analysis (Fig. 7a). Elevated levels of *Pds* mRNA in leaf tissues was detected in all of them (Fig. 7b). Measurement of carotenoids in the leaves demonstrated increase of 24% in total carotenoids content and no change in the chlorophyll/carotenoid ratio (Table I).

It was concluded that for altering expression of *Pds*, low level expression from a regulated promoter is required.

Figure 7: (a) Southern DNA analysis of genomic DNA from different tobacco plants carrying the vector pBOX, over-expressing *Pds* (OX-2,3,4,7,8) and of plants carrying just the plasmid pBIB (C-8,9). The DNA was digested with HindIII and was hybridized to the nptII sequence. **(b)** RNA blot analysis (Northern) of the above plants. Antisense RNA of *Pds* was used as a molecular probe.

Table I: Carotenoids and chlorophyll content in transgenic tobacco plants over-expressing the mutated *Pds* gene and in control plants (wild-type, wt, and Ti-transformed plants).

Over-expression:

Plant No.	Carotenoids [$\mu\text{g}/\text{mgFW}$]	Chlorophyll [$\mu\text{g}/\text{mgFW}$]	chlorophyll/carotenoids
OX-2	0.34 ± 0.02	0.95 ± 0.06	0.362 ± 0.004
OX-3	0.33 ± 0.01	0.94 ± 0.01	0.358 ± 0.004
OX-4	0.34 ± 0.01	0.95 ± 0.0	0.356 ± 0.003
OX-8	0.34 ± 0.02	0.93 ± 0.06	0.360 ± 0.001
Average	0.34 ± 0.01	0.94 ± 0.03	0.359 ± 0.003

Control plants:

w.t.	0.24 ± 0.01	0.74 ± 0.02	0.328 ± 0.004
C-7	0.32 ± 0.01	0.88 ± 0.02	0.359 ± 0.001
C-8	0.23 ± 0.04	0.70 ± 0.12	0.322 ± 0.002
Average	0.27 ± 0.04	0.79 ± 0.09	0.338 ± 0.002

Genetic engineering of herbicide resistance

The tomato cDNA of *Pds* was changed by site-directed mutagenesis at codon 512 from GTT (Val) to GGT (Gly). A similar mutation in the homologous site (residue 403) of the *Synechococcus* sp. PCC7942 *pds* gave rise to norflurazon resistance. The mutated cDNA was fused with the 35S CaMV promoter in the Ti vector pBIB, transfected to *A. tumefaciens* strain LBA4404 and transgenic tobacco plants were regenerated following infection. Several of these regenerants showed co-suppression phenotype as described above in the over-expression experiments.

However, some transgenic plants had normal appearance and produced seeds. These plants, OM1-5, were further analyzed. Southern and northern hybridization confirmed the presence of *Pds* and its expression in the transgenic plants (Fig. 8). Carotenoid analysis indicated that the mutated gene was functional, namely, in spite of the amino acid substitution in *Pds*, an increase in total carotenoids has been observed, similarly to the wild-type *Pds* transformed plants (Table II).

Figure 8: (a) Southern DNA analysis of genomic DNA from different tobacco plants carrying the vector pBOX^m, over-expressing the mutated *Pds* (V512G) (OM-1,2,3,4,5). The DNA was digested with HindIII and was hybridized to the *nptII* sequence. (b) RNA blot analysis (Northern) of the above plants. Antisense RNA of *Pds* was used as a molecular probe.

Table II: Carotenoids and chlorophyll content in transgenic tobacco plants over-expressing the mutated *Pds* gene (V512G).

Plant No.	Carotenoids [$\mu\text{g}/\text{mgFW}$]	Chlorophyll [$\mu\text{g}/\text{mgFW}$]	chlorophyll/carotenoids
OM-1	0.32 ± 0.01	0.89 ± 0.03	0.358 ± 0.002
OM-2	0.31 ± 0.01	0.85 ± 0.01	0.362 ± 0.003
OM-3	0.25 ± 0.01	0.77 ± 0.04	0.323 ± 0.001
OM-4	0.30 ± 0.01	0.85 ± 0.03	0.354 ± 0.001
OM-5	0.33 ± 0.01	0.89 ± 0.02	0.367 ± 0.003
Average	0.30 ± 0.03	0.85 ± 0.05	0.353 ± 0.002
Average of control plants	0.27 ± 0.04	0.79 ± 0.09	0.338 ± 0.002

Norflurazon resistance was assayed by germinating seeds on MS-agar plates containing various concentrations of norflurazon. At concentrations of 100-200 ng the wild-type seedlings were bleached while the transgenic plants were tolerant to the herbicide (Fig. 9). However, at 250 ng the transgenic plants were bleached too. The narrow margin of resistance to norflurazon in these transformants cannot be used as a selectable marker. However the results sustain the rationale of the experiment and warrant further attempts in this direction.

Control (MS medium)

100 nM norflurazon

250 nM norflurazon

OM-2

control (OX-3)

Figure 9: Transgenic tobacco seedlings of transformant OM-2, expressing the mutated *Pds* grown on MS medium which contains the indicated concentration of norflurazon.

Cloning the cDNA of lycopene ϵ -cyclase

The enzyme lycopene ϵ -cyclase operates in the biosynthetic pathway of α -carotene (and lutein) from the linear carotene lycopene. We have cloned the cDNA for this enzyme from tomato (*L. esculentum*) using a cDNA for lycopene ϵ -cyclase that has been identified in *Arabidopsis* (kindly provided by Dr. F.X. Cunningham).

To verify the identity of this cDNA we have inspected the activity of its polypeptide product in *E. coli* cells. For this purpose we have constructed the following plasmids (Fig. 10): 1) pACCRT-EIB, which carries the genes *crtE*, *crtB* and *crtI* from *Erwinia uredvora* on the pACYC184 vector. *E. coli* cells that carry this plasmid produce and accumulate lycopene; 2) pCRTL-e, a pBluescript which carries the cDNA for ϵ -cyclase from tomato; 3) pCRTL-b, a pBluescript which contains the cDNA of lycopene β -cyclase; 4) pBCAR, which is essentially similar to pACCRT-EIB, but with the genes from *Erwinia herbicola* plus the *crtY* gene. *E. coli* cells which contain this plasmid produce β -carotene (Fig. 10).

Figure 10: Schematic structure of plasmids pACCRT-EIB, pCRTL-e, pCRTL-b and pBCAR.

Expression of the cDNA of *CrtL-e* in *E. Coli* cells that produce lycopene, resulted in the accumulation of mainly the single-ring molecule of δ -carotene, and only little amount of ϵ -carotene was formed (Fig. 11 and Table III). This is in contrast to the lycopene β -cyclase which introduces two β -ionone rings in each end of the linear molecule of lycopene. Expression of both *CrtL-b* and *CrtL-e* in *E. Coli* cells that produce lycopene, resulted in the synthesis of α -carotene (Table III). These results confirm that *CrtL-e* codes for lycopene ϵ -cyclase, which catalyzes the formation of a single ϵ -ionone ring on the linear molecule of lycopene. This is in contrast to the β -cyclase that introduces two β -ionone rings to lycopene. In addition, the results indicate that α -carotene is synthesized by the two lycopene cyclases and the same lycopene β -cyclase functions in the biosynthesis of both β -carotene and α -carotene. The formation of β -carotene and α -carotene is depicted in Fig. 12.

Figure 11: Analysis by HPLC of carotenoids accumulated in *E. coli* cells carrying plasmid pACCRT-EIB (A) or pACCRT-EIB + pCRTL-E (B). Peaks of the HPLC: 1- lycopene; 2- δ -carotene; 3- ϵ -carotene. Identification of the peaks was based on their typical retention time and absorbance spectra (in boxes). AU- arbitrary units.

Table III: Accumulation of carotenoids in *E. Coli* cells, which carry the plasmid pACCRT-EIB, expressing *CrtL-b* and *CrtL-e* from tomato [percent of total carotenoids].

	lycopene	δ -carotene	β -carotene	α -carotene	ϵ -carotene
<i>CrtL-b</i>	2	-	98	-	-
<i>CrtL-e</i>	2.1	85.5	-	-	12.4
<i>CrtL-b</i> + <i>CrtL-e</i>	-	-	67	33	-

Figure 12: Enzymatic steps leading to the biosynthesis of the major xanthophylls in plants. In contrast to β -carotene, which is produced from lycopene by a single enzyme, lycopene β -cyclase, α -carotene is made by two enzymes, lycopene β -cyclase and lycopene ϵ -cyclase.

Sequence analysis, phylogeny and evolution of the lycopene cyclase genes

Sequence analysis of the tomato cDNA for lycopene ϵ -cyclase, termed *CrtL-e*, revealed a polypeptide of 526 amino-acid residues with a molecular weight of 58.8 kDa (Fig. 13). A transit sequence of 106 residues for chloroplast import can be recognized in the amino terminus of the protein. The primary structure of *CRTL-e* from tomato is 72% identical and 93% similar with its homologous protein from *Arabidopsis* and 36% identical and 78% similar to the tomato lycopene β -cyclase, *CRTL-b* (Fig. 13,14). Surprisingly, *CRTL-e* is also similar to the enzyme capsanthin-capsorubin synthase, which converts antheraxanthin and violaxanthin to capsanthin and capsorubin in pepper fruits. All of these enzymes contain a conserved dinucleotide-binding motif (FAD) in the amino terminus of the mature polypeptide. Three additional conserved sequences that are found also in bacterial lycopene cyclases have been identified (Fig. 15).

We have used the parsimony method of PHYLIP (Phylogeny Interface Package) version 3.5c (Distributed by J. Felsenstein) to analyze the amino acid sequences of different lycopene cyclases and CCS. The results are depicted as an unrooted phylogenetic tree (Fig. 16). It is hypothesized that *CrtL-e* evolved from *CrtL-b* in eukaryotic organisms (algae) and that CCS evolved from *CrtL-e* in land plants.

1	1	TT	GAA	M	E	C	V	G	V	Q	N	V	G	A	M	A	V	L	T	R	P
19	60	AGA	TTG	N	R	W	S	G	G	E	L	C	Q	E	K	S	I	F	L	A	Y
39	120	GAG	CAG	TAT	GAA	AGT	AAA	TGT	AAT	AGC	AGT	AGT	GGT	AGT	GAC	AGT	TGT	GTA	GTT	GAT	AAA
59	180	GAA	GAT	TTT	GCT	GAT	GAA	GAA	GAT	TAT	ATA	AAA	GCC	GGT	GGT	TCG	CAA	CTT	GTA	TTT	GTT
79	240	CAA	ATG	CAG	CAG	AAA	AAA	GAT	ATG	GAT	CAG	CAG	TCT	AAG	CTT	TCT	GAT	GAG	TTA	CGA	CAA
99	300	ATA	TCT	GCT	GGA	CAA	ACC	GTA	CTG	GAT	TTA	GTG	GTA	ATC	GGC	TGT	GGT	CCT	GCT	GGT	CTT
119	360	GCT	CTT	GCC	GCG	GAG	TCA	GCT	AAA	TTG	GGG	TTG	AAC	GTG	GGG	CTC	GTT	GG	CTT	GAT	CTT
139	420	CCT	TTC	ACA	AAC	AAC	TAT	GGT	GTA	TGG	GAG	GAC	GAG	TTC	AAA	GAT	CTT	GGT	CTT	CAA	GCC
159	480	TGC	ATT	GAA	CAT	GTT	TGG	CGG	GAT	ACC	ATT	GTA	TAT	CTT	GAT	GAT	GAT	GAA	CCT	ATT	CTT
179	540	ATT	CCC	CGT	GCC	TAT	GGA	AGA	GTT	AGT	CGC	CAT	TTT	CTG	CAC	GAG	GAG	TTA	CTC	AAA	AGG
199	600	TGT	GTG	GAG	GCA	GGT	GTT	LT	Y	TAT	CTA	AAC	TCG	AAA	GTG	GAT	AGG	ATT	GTT	E	ACA
219	660	AAT	GGC	CAG	AGT	CTT	GTA	GAG	TGC	GAA	GGT	GAT	GTT	GTG	ATT	CCC	TGC	AGG	TTT	GTG	ACT
239	720	GTT	GCA	TCG	GGG	GCA	GCC	TCG	GGG	AAA	TTC	TTG	CAG	TAT	GAG	TTG	GGA	AGT	CCT	AGA	GTT
259	780	TCT	GTT	CAA	ACA	GCT	TAT	GGA	GTG	GAA	GTT	GAG	GTT	GAT	AAC	AAT	CCA	TTT	GAC	CCG	AGC
279	840	CTG	ATG	GTT	TTC	ATG	GAT	TAT	AGA	GAT	TAT	CTC	AGA	CAC	GAC	GCT	CAA	TCT	TTA	GAA	GCT
299	900	AAA	TAT	CCA	ACA	TTT	CTT	TAT	GCC	ATG	CCC	ATG	TCT	CCA	ACA	CGA	GTC	TTT	TTT	GAG	EAA
319	960	ACT	TGT	TTG	GCT	TCA	AAA	GAT	GCA	ATG	CCA	TTC	GAT	CTG	TTA	AAG	AAA	AAA	CTG	ATG	CTA
339	1020	CGA	TTG	AAC	ACC	CTT	GGT	GTA	AGA	ATT	AAA	GAA	ATT	TAC	GAG	GAG	GAA	TGG	TCT	TAC	ATA
359	1080	CCG	GTT	GGT	GGA	TCT	TTG	CCA	AAT	ACA	GAA	CAA	AAA	ACA	CTT	GCA	TTT	GGT	GCT	GCT	GCT
379	1140	AGC	ATG	GTT	CAT	CCA	GCC	ACA	GGT	TAT	TCA	GTC	GTC	AGA	TCA	CTT	TCT	GAA	GCT	CCA	AAA
399	1200	TGC	GCC	TCT	GTA	CTT	GCA	AAT	ATA	TTA	CGA	CAA	CAT	TAT	AGC	AAG	AAC	ATG	CTT	ACC	AGT
419	1260	TCA	AGT	ATC	CCG	AGT	ATA	TCA	ACT	CAA	GCT	TGG	AAC	ACT	CTT	TGG	CCA	CAA	GAA	CGA	AAA
439	1320	CGA	CAA	AGA	TCG	TTT	TTC	CTA	TTT	GGA	CTG	GCT	CTG	ATA	TTG	CAG	CTG	GAT	ATT	GAG	GGG
459	1380	ATA	AGG	TCA	TTT	TTC	CGC	GCA	TTC	TTC	CGT	GTG	CCA	AAA	TGG	ATG	TGG	CAG	GGA	TTT	CTT
479	1440	GGT	TCA	AGT	CTT	TCT	TCA	GCA	GAC	CTC	ATG	TTA	TTT	GCC	TTC	TAC	ATG	TTT	ATT	ATT	GCA
499	1500	CCA	AAT	GAC	ATG	AGA	AAA	GGC	TTG	ATC	AGA	CAT	CTT	TTA	TCT	GAT	CCT	ACT	GGT	GCA	ACA
519	1560	TTG	ATA	AGA	ACT	TAT	CTT	ACA	TTT	TAG	AGT	AAA	CTC	CTC	CTA	CAA	TAA	TTG	TTG	AAT	CAG
1620		AGG	CCT	CAT	TAC	TTC	AGA	TTC	ATA	ACA	GAA	ATC	GCG	GTC	TCT	CGA	GGC	ATT	GTA	TAT	AAC
1680		ATT	TTC	ACT	AGC	TTA	ATA														

Figure 13: Nucleotide sequence and deduced amino acid sequence of the cDNA of the tomato *CRTL-e*.

Figure 14: Comparison of amino acid sequences of lycopene cyclases from different organisms. Epstom- ϵ -cyclase from tomato; Epsarab- ϵ -cyclase from *Arabidopsis*; Crtltom- β -cyclase from tomato; Syn7249- β -cyclase from the cyanobacterium *Synechococcus* PCC7942; CrtYeu- lycopene cyclase (β) from *Erwinia uredovora*.

Fig. 16. Unrooted phylogenetic tree of lycopene cyclase enzymes from cyanobacteria and plants. *CrtL-b*, lycopene β -cyclase; *CrtL-e*, lycopene epsilon cyclase; *CCS*, capsanthin capsorubin synthase.

Expression of *CrtL-b* *CrtL-e* during tomato fruit ripening

The carotenoid composition in the green stages of fruit ripening in tomato is similar to that of green leaves namely, lutein, violaxanthin, neoxanthin and β -carotene predominate. At the breaker stage lycopene begins to accumulate and reaches up to 700 fold increase in the red stage.

As we (shown above) and other groups have shown, transcription of the genes for early enzymes in the carotenoid biosynthesis pathway - phytoene synthase (*Psy*) and phytoene desaturase (*Pds*), is increased during fruit ripening in tomato. Recently, we have discovered that the mRNA level of *CrtL- β* , which encodes the lycopene β -cyclase, decreases during fruit ripening.

To determine the regulation of expression of *CrtL-e* during tomato fruit development we have measured by RT-PCR its relative mRNA levels at different stages of ripening. It was found that, similarly to *CrtL- β* , the level of mRNA of *CrtL- ϵ* decreases during ripening (Fig. 17).

To determine the regulation of expression of *CrtL-e* during tomato fruit development we have measured by RT-PCR its relative mRNA levels at different stages of ripening. It was found that, similarly to *CrtL-β*, the level of mRNA of *CrtL-ε* decreases during ripening (Fig. 17).

These results indicate that regulation of expression, most probably at the level of transcription, of genes for the carotenoid biosynthesis enzymes plays a major role in the accumulation of lycopene in tomato fruits. While the mRNA of lycopene biosynthesis enzymes is up-regulated, the expression of the genes for the two enzymes which utilize lycopene for further biosynthetic steps is down-regulated.

The overall regulation of mRNA levels of carotenoid genes is illustrated in Fig. 18.

Figure 17: Regulation of *CrtL-e* and *Pds* expression during tomato fruit ripening. mRNA levels of *CrtL-e* and *Pds* were determined by RT-PCR of total cellular RNA. Equal amounts of total RNA were included in each reaction. As a control for the linearity of the amplification, 1/3 and 3x amount of total leaf RNA were determined (lanes 1 and 2). The PCR-products were radioactively labeled during the amplification, separated by polyacrylamide gel electrophoresis and autoradiographed. Amplification of mRNA of pre-albumin served as a control of the RT-PCR reaction (control). l- leaf; ig- immature green; mg- mature green; b- breaker; o- orange; p- pink; r- red.

Figure 18: Levels of mRNA in tomato fruits during ripening (relative units). Ripening stages: G, green; MG, mature green; B, breaker; O, Orange; P, pink; R, red.

Expression of *Pds* and *CrtL-e* during fruit ripening in the color mutant *Del*

To determine the regulation of expression of *CrtL-e* during fruit development in tomato, we have measured by RT-PCR its mRNA level at different stages of ripening (Fig. 19). The result indicated *Pds* is up-regulated, as expected, but in contrast to the “wild-type” *L. esculentum*, the mRNA of *CrtL-e* in the fruit of the *Delta* mutant increases dramatically at the breaker stage of ripening. This result provides an explanation for the accumulation of δ -carotene in the fruits of the *Delta* mutant. Since *CrtL-e* maps to the *Del* locus, it is very likely that the difference between the “wild-type” and the *Delta* is in the promoter region of *CrtL-e*. This hypothesis is supported by the fact that the origin of *Del* in *L. Esculentum* is from a wild species.

Figure 19: Regulation of *CrtL-e* and *Pds* expression during tomato fruit ripening in the color mutant *Delta*. mRNA levels of *CrtL-e* and *Pds* were determined by RT-PCR of total cellular RNA as described in Fig. 18. l- leaf; ig- immature-green; mg- mature-green; 0- orange; p- pink; r- red.

Immunological detection of phytoene desaturase

Polyclonal antibodies were raised against *PDS* by immunization of rabbits with a LACZ-PDS fusion protein produced by *E. coli* carrying pPDSdel35 (Fig. 20). The polypeptide band was identified by SDS-polyacrylamide gels (Fig. 21). The anti-PDS antiserum recognized the LACZ-PDS fusion protein as well as a smaller fusion protein produced by *E. coli* cells carrying the deleted LACZ-PDS fusion protein produced by pPDSdel52. Neither of these bands were detected in protein extracts of *E. coli* cells not carrying these plasmids, or by the preimmune serum (results not shown). The antibodies recognized a protein in *Synechococcus* sp. PCC 7942 of an apparent molecular weight of 50 kDa (Fig. 22). This size of a protein corresponds well to the predicted molecular weight of the PDS. In tomato plants these antibodies reacted with a polypeptide of 51 kDa only when chloroplast membranes were prepared and their polypeptides were separated by SDS-PAGE. This reaction was too feeble to allow definite detection of *PDS* in electron microscopical analysis. Attempts at getting better antibodies against the tomato *PDS* have been futile because of technical problems in purification of this type of *PDS* from *E. coli*. Hence, we decided to end this line of investigation .

Figure 20: Structure of plasmid pPDSdel35PYS, expressing *PDS* in *E. coli*.

Figure 21: Coomassie blue stained polyacrylamide gels of total proteins from *E. coli* cells carrying plasmid pPDSdel35 (A); or plasmid pPDSdel52 (B). Arrows indicate over-expression of *PDS* in each construct.

Figure 22: Western blot analysis with anti-PDS antibodies of proteins from: *E. coli* cells carrying plasmid pPDSdel35 (A); *E. coli* cells carrying plasmid pPDSdel52 (B); or cyanobacterial total membrane proteins (C-E).

METHODS

Cloning and sequence analysis

A cDNA Library of leaf RNA from *Lycopersicon esculentum* cv. VF36, in the lambda phage vector gt10, was kindly provided by Dr. S. Tanksley from Cornell.

Cloning in plasmid vectors in *E. coli* XLI Blue cells and screening of recombinant plasmids were according to standard protocols. DNA hybridization was done at low stringency in a buffer containing 35% formamid, 5x standard saline citrate (SSC), 5x Denhart's solution, 0.2% SDS, at 37°C. Filters were washed with 2x SSC at 37°C.

Sequence analysis was performed with the Sequenase II kit of United States Biochemical Corporation. Overlapping fragments were subcloned and both strands of each fragment were sequenced. Nucleotide sequence analyses and comparisons were done using the UWGCG software package and amino acid comparisons were done by HIBIO PROSIS (Hitachi software Engineering, Tokyo).

Pigment extraction and analysis

E. coli cells of the strain XLI blue, carrying carotenoid biosynthesis genes from *Erwinia herbicola*, *E. uredoovora* and *L. esculentum* in various plasmid vectors, accumulate carotenoids.

For pigment extraction 2 ml of an over night culture was harvested by centrifugation and the pellet was resuspended in 200 μ l of acetone. The samples were incubated at 65°C for 30 minutes, centrifuged at 13,000 rpm and the supernatant was analyzed by HPLC. 50 μ l aliquots were injected into a 3.2 mm x 25 cm reverse phase C18 Spherisorb column, ODS2 (5 μ m particle size). The mobile phase consisted of acetonitrile:H₂O (9:1) - solvent A and 100% ethyl acetate - solvent B. The pigments were separated by a linear gradient between solvent A and solvent B for 25 minutes, at flow of 1 ml.min⁻¹. The solvents were pumped by a Merck-Hitachi L-600A high pressure pump. Peaks were detected at 450 nm by a Merck-Hitachi diode-array detector L-4500. Carotenoids were identified by their characteristic absorption spectra and their typical retention time, which corresponded to standard compounds of neurosporene, lycopene and β -carotene. Areas of peaks were integrated by the Merck-Hitachi HPLC software and quantified against a standardization equation derived by injection of known quantities of each pigment.

Extraction of RNA

Tomato fruits were crashed in a blender in 2.5 ml/gr buffer containing 4 M guanidine thiocyanate, 0.1 M Na-acetate pH 5.2, 0.7% β -mercaptoethanol, and then homogenized by polytron. Sarcosyl was added to a final concentration of 1% and the homogenate was centrifuged and filtered through Mira cloth. Two milliliters of water-saturated phenol were added, mixed well and then 0.5 ml chloroform were added. The samples were centrifuged and the aqueous phase was collected and precipitate with an equal volume of isopropanol. The pellet was resuspended in 1 ml water and 0.2 ml ethanol were added and following 30 minutes on ice the samples were centrifuged and the supernatant was transferred to a new tube and precipitated with 0.33 volumes of 8 M LiCl. Following centrifugation the pellet was washed with 80% ethanol, dried and resuspended in water. RNA concentration and purity were determined by spectrophotometry and its integrity was examined by electrophoresis on formaldehyde gels and ethidium bromide staining. Leaf RNA was extracted using a similar procedure, tissue was ground in liquid nitrogen, the first stages up to the chloroform extraction were combined, excluding the filtration. Alternatively leaf RNA was extracted using the TRI REAGENT protocol (Molecular Research Center, Inc., Cincinnati, OH).

Measurement of mRNA by RT-PCR

Reverse transcription was carried out using 25 ng of total RNA and oligo dT as a primer. Concentration of total plant RNA was determined by spectrophotometrical analysis. All RNA samples in each experiment were analyzed by separation in formaldehyde agarose gel electrophoresis and visual inspection of the rRNA bands seen following ethidium bromide staining. The reaction mixture included 1 mM dNTP's, 0.5 μ M oligo dT, 20 units RNase, 100 ng of control RNA (from rat hepatoma cell line HP4), 10 mM DTT, 1x RT buffer (BRL) and M-MLV reverse transcriptase (BRL) in a total volume of 20 μ l. The reaction mixture was incubated for 10 minutes at room temperature and then 40 minutes at 37°C.

The PCR reaction mixture contained 0.625 μ M of each primer, 1x Taq polymerase buffer, Taq polymerase and the entire RT reaction mixture in a total volume of 80 μ l. The RT reaction mixture was incubated for 5 minutes at 95°C prior to addition to the PCR reaction mixture. The amplification reactions included 26 cycles of 1 minute at 95°C, 1 minute at 60°C and 1 minute at 72°C. To define the linear stage of the reaction samples were taken following

22 cycles and every two cycles up to 32. Different quantities of RNA were used in order to demonstrate a linear ratio between the template mRNA and the final PCR product. The primers used for amplification of *Pds-1* were: 5'-TTGTGTTTGCCGCTCCAGTGGATAT-3' at position 1127, and 5'-GCGCCTTCCATTGAAGCCAAGTAT-3' at position 1652. The primers used for amplification of *CrtL-b* were: 5'-GGCTTCTCTAGATCTCTTGTTTCAG-3' at position 760 and 5'-GTTTCAGGTAGAAACAATCGAGACG-3' at position 1440. The primers used for amplification of *CrtL-e* were: 5'-ATTTGACCCGAGCCTGATGG-3' at position 827 and 5'-ATGCCTCGAGAGACCGCG-3' at position 1440. Primers for amplification of the rat pre-albumin cDNA which were used as a control of the RT-PCR reactions were: 5'-AGTCCTGGATGCTGTCCGAG-3' at position 131 and 5'-TTCCTGAGCTGCTAACACGG-3' at position 571. Quantitation of PCR products was carried out by including 2 μ Ci of ^{35} S-dATP in the PCR reaction. PCR products were separated by electrophoresis on acrylamide gel. Following electrophoresis, the gel was dried and, monitored by phosphorimager (BAS1000 - FUJIX) and then exposed to an X-ray film. The results were corrected according to the relative quantity of the control PCR product.

Immunological protocols

Antibodies against PDS were prepared in rabbits with a polypeptide that was extracted from *E. coli* cells carrying plasmid pPDSdel35. plasmid expressed *Pds* from *Synechococcus* PCC 7942. The polypeptide band was identified by SDS-polyacrylamide gels, from which it was then extracted, homogenized with an equal volume of Freund's adjuvant, and injected subcutaneously into rabbits. Boosters of the fusion protein were administered every two weeks as needed. Serum was collected two weeks after each booster.

To extract membrane proteins from the cyanobacteria, 100 ml of a culture in logarithmic phase of growth were harvested, washed and resuspended in 1 ml of phosphate buffered saline (PBS). 300 μ l of glass beads (200 μ m diameter) were added and the cells disrupted in a Brown homogenizer. The suspension was centrifuged at 5000 rpm for ten minutes and the supernatant transferred to a new tube. Membranes were isolated by centrifugation in a microfuge at 15,000 rpm for 30 minutes. The membrane pellet was resuspended in PBS.

Amount of 25 μ g of membrane proteins were separated by SDS-PAGE (Laemmli 1970) through a 12.5% acrylamide gel. Proteins were transferred from polyacrylamide gels to

polyvinylidene fluoride (PVDF) filters (Millipore) by electro-blotting. The filter was blocked by incubation for two hours with 5% nonfat milk and 0.2% Tween-20 in PBS. The filter was incubated with the antiserum diluted 1:500 in the blocking buffer. Cell lysate of *E. coli* (Sambrook et al. 1989) strain HB101 from a 10 ml stationary suspension culture was added to the incubation mixture to absorb non-specific anti-*E. coli* antibodies. Detection of bands corresponding to PDS was augmented with goat anti-rabbit immunoglobulin G-alkaline phosphatase following signal enhancement with the BLAST kit (DuPont).

CONCLUSIONS

The results that have been obtained in this research establish a paradigm for the regulation of carotenoid pigment biosynthesis during the ripening process of fruits. Together with previous data it is evident that transcriptional regulation of genes that encode carotenoid-biosynthesis enzymes is the major mechanism that governs specific pigment accumulation. These findings support the working hypothesis of the molecular approach to manipulating carotenogenesis by altering gene expression in transgenic plants, and suggests clear approaches to future application in agriculture.

A novel gene encoding lycopene ϵ -cyclase in tomato has been cloned and molecularly analyzed.

The molecular basis for the tomato color mutation *Delta* has been determined.

Attempt to generate herbicide resistance in transgenic plants using a mutated *Pds* has been made. However, this approach yielded only partial resistance of no practical application.

REFERENCES

1. Grierson, D. and Schuch, W. (1993) Philos. Trans. R. Soc. Lond. [Biol]. 342, 241-250.
2. Gillaspay, G., Ben-David, H. and Gruissem, W. (1993) Plant Cell 5, 1439-1451.
3. Pecker, I., Gabbay, R., Cunningham, F.X. and Hirschberg, J. (1996) Plant Mol. Biol. 30, 807-819.
4. Ray, J.A., Bird, C.R., Maunders, M., Grierson, D. and Schuch, W. (1987) Nucleic Acids Res. 15, 10587-10588.
5. Ray, J.A., Moureau, P., Bird, A.S., Grierson, D., Maunders, M., Truesdale, M., Bramley, P.M. and Schuch, W. (1992) Plant Mol. Biol. 19, 401-404.
6. Corona, V., Aracri, B., Kosturkova, G., Bartley, G.E., Pitto, L., Giorgetti, L., Scolnik, P.A. and Giuliano, G. (1996) Plant J. 9, 505-512.

DESCRIPTION OF COOPERATION

Analysis of *Pds* expression during fruit ripening in tomato and construction of various DNA vectors for expression in plants were done in Jerusalem. Improvement of transformation protocols for tomato and citrus and construction of cDNA library from grapefruit were done in Gainesville. The cDNA of *CrtL*, encoding lycopene cyclase from tobacco, has been sent to Florida to be used as a probe in cloning its homolog from grapefruit. Strains of *E. coli* that carry specific cDNA species, which have been identified by "color complementation" screen in Gainesville, were sent to Jerusalem where their carotenoids have been analyzed by HPLC (no color changes have been found). This is a first step towards manipulating fruit color in citrus.

EVALUATION OF THE RESEARCH ACHIEVEMENTS WITH RESPECT TO THE ORIGINAL RESEARCH PROPOSAL AND ITS OBJECTIVES

The overall goal of this research was to study carotenoid biosynthesis at the molecular level in order to eventually be able to manipulate fruit color (and carotenoid composition) through genetic transformation techniques utilizing the cloned carotenoid biosynthesis genes - *Pds* and *Psy*.

Specific goals of the research were to study the regulation of expression of *Pds* and to produce resistance to "bleaching herbicides" in transgenic plants that contain a genetically modified *Pds*. The research plan of the original proposal outlined the following seven specific aims:

1. Studying the expression of *pds* during fruit ripening.
2. Immunological detection of phytoene desaturase.
3. Analyzing the expression of *pds* in fruit color mutants.
4. Genetic transformation of tomato.
5. Altering *pds* expression in tomato.
6. Promoter analysis of *pds*.
7. Developing herbicide-resistance in plants.

All the above topics have been investigated. All of them, except aim #2, provided novel results of utmost importance and significance for future application in plant breeding and plant biotechnology. As explained above, work on *Psy* has not been pursued because this part has been carried out in-, and published by another laboratory. Instead, we have expanded the investigation beyond its original plan and added new goals. We have cloned the gene *CrtL-e*, which encodes lycopene ϵ -cyclase, and analyzed the regulation of its expression during fruit ripening.

The major and most significant outcome of this research is the explanation for the

accumulation of lycopene in tomato fruits (“why tomatoes are red”). Our results indicate that transcriptional regulation of carotenoid biosynthesis genes is the key mechanism for over-production and accumulation of lycopene. During the ripening process the transcription of genes encoding the enzymes phytoene synthase and phytoene desaturase is up-regulated, while the transcription of the genes for both lycopene cyclases decreases and thus the conversion of lycopene to subsequent carotenoids is inhibited.

The benefit for agriculture of this research program is quite obvious. Carotenoids are natural pigments that are responsible for many of the yellow, orange and red colors seen in many plants. In flowers and fruits they facilitate the attraction of pollinators and dispersal of seeds. This latter aspect is strongly associated with agriculture since the type and degree of pigmentation in fruits and flowers are among the most important traits of many crops. This is mainly since the colors of these products often determine their appeal to the consumers and thus can increase their market worth. Since carotenoids are effective antioxidants *in vivo* and also serve as provitamin A, they have a high nutritious value to humans. Therefore, it is desirable to increase the concentration of specific carotenoids, such as β -carotene, in fresh agricultural products. In addition, many carotenoids have important industrial and pharmaceutical uses as coloring agents of food and cosmetics.

The molecular and physiological knowledge on carotenogenesis during fruit ripening, which has been gained in this project, suggest a concept for manipulating gene expression that will alter carotenoid composition in fruits and flowers.

PUBLICATIONS RESULTING FROM THE RESEARCH PROJECT

Mann, V., Pecker, I. and Hirschberg, J. (1994) Cloning and characterization of the gene for phytoene desaturase (*Pds*) from tomato (*Lycopersicon esculentum*). **Plant Mol. Biol.**, 24:429-434.

Hirschberg, J., Pecker, I., Lotan, T., Gabbay, R., and Mann, V. (1995) Regulation of carotenoid biosynthesis in plants and algae. In: "**Photosynthesis: From Light to Biosphere,**" Vol. IV, Mathis, P. ed., Kluwer Academic Publishers, pp. 9-14.

Hirschberg, J., Cohen, M., Harker, M., Lotan, T., Mann, V. and Pecker I. (1997) Molecular genetics of the carotenoid biosynthesis pathway in plants and algae. **Pure Appl. Chem.** (In Press)

Ronen G, Cohen M, Zamir D & Hirschberg J. Molecular analysis and genetic mapping of the cDNA for lycopene epsilon-cyclase from tomato (submitted for publication).