MARCIO GILBERTO CARDOSO COSTA

MORFOGÊNESE *in vitro*, TRANSFORMAÇÃO GENÉTICA, CLONAGEM E SUPEREXPRESSÃO DE GENES DA ROTA BIOSSINTÉTICA DE CAROTENÓIDES EM CITROS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do título de *Doctor Scientiae*

VIÇOSA
MINAS GERAIS - BRASIL
2002

MARCIO GILBERTO CARDOSO COSTA

MORFOGÊNESE *in vitro*, TRANSFORMAÇÃO GENÉTICA, CLONAGEM E SUPEREXPRESSÃO DE GENES DA ROTA BIOSSINTÉTICA DE CAROTENÓIDES EM CITROS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do título de *Doctor Scientiae*

APROVADA: 21 de junho de 2002.

Prof. Carlos Roberto de Carvalho

Prof. Raimundo Santos Barros

Prof. Sérgio H. Brommonschenkel
(Conselheiro)

Prof. Sílvio Lopes Teixeira

Prof. Wagner Campos Otoni
(Orientador)

Aos meus pais, Gilberto M. Costa e Maria da Graça C. Costa.

À minha esposa, Silvia Kimo Costa.

Ao meu filho, Lucas Kimo Costa.

AGRADECIMENTO

À Universidade Federal de Viçosa, pela oportunidade de realização deste curso.

À Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pelo apoio financeiro dado no Brasil e nos Estados Unidos (processo # BEX0600/00-8).

Aos meus pais, à minha esposa e ao meu filho, pelo incentivo e amor dados ao longo dessa jornada.

Ao Prof. Wagner Campos Otoni, pela orientação, pela dedicação incansável em todas as fases deste trabalho, pelo exemplo de conduta e, sobretudo, pela amizade e confiança.

À Dra. Gloria A. Moore, pela oportunidade de realização de parte deste curso na Universidade da Flórida (Gainesville, FL, EUA), pelos sábios ensinamentos, pela amizade e confiança.

Aos colegas de trabalho na Universidade da Flórida Eduardo F. Carlos, Elvia C. Palácios Torres, Karen I. Champ, Kimberly Ruesch, Maureen Meyerson, Mehtap Sahin-Çevik, Ufuk Koca, Vicente J. Febres e Yavuz Yagiz, pela ajuda, pelo companheirismo e pela amizade.

Ao Dr. Walter dos Santos Soares Filho (EMBRAPA/CNPMF, Cruz das Almas, Ba), pelo gentil fornecimento de sementes de laranja e pomelo para o desenvolvimento de parte desse trabalho.

Aos professores Carlos Roberto de Carvalho, Luiz Orlando de Oliveira, Paulo Roberto Mosquim, Raimundo Santos Barros, Sérgio Hermínio Brommonschenkel e Sílvio Lopes Teixeira, pelas importantes contribuições dadas ao trabalho e à minha formação.

A todos os professores que contribuíram para o meu crescimento profissional e humano.

Aos colegas de curso, pela convivência agradável.

BIOGRAFIA

MARCIO GILBERTO CARDOSO COSTA, filho de Gilberto Machado Costa e Maria da Graça Cardoso Costa, nasceu em São Sepé, RS, no dia 15 de julho de 1973.

Concluiu seus estudos de primeiro e segundo graus naquela cidade.

Em julho de 1991, ingressou na Universidade Federal de Santa Maria, Santa Maria-RS, graduando-se em Agronomia em julho de 1996.

No período de abril de 1995 a agosto de 1996, foi monitor das disciplinas Melhoramento Vegetal e Melhoramento Florestal, do Departamento de Fitotecnia da Universidade Federal de Santa Maria, no qual também desenvolveu atividades de pesquisa na área de cultura de tecidos vegetais.

Em outubro de 1996, iniciou o Mestrado em Genética e Melhoramento, na Universidade Federal de Viçosa, Viçosa-MG, defendendo tese em outubro de 1998.

Em outubro de 1998, iniciou o Doutorado em Genética e Melhoramento, na Universidade Federal de Viçosa, defendendo tese em junho de 2002.

No período de setembro de 2000 a março de 2002, esteve no Departamento de Ciências Horticulturais da Universidade da Flórida, Gainesville-FL, EUA, desenvolvendo parte da tese de doutorado de acordo com o programa de Doutorado-Sanduíche.

CONTEÚDO

Página

RESUMO x

ABSTRACT xii

INTRODUÇÃO GERAL 1

REFERÊNCIAS BIBLIOGRÁFICAS 4

CAPÍTULO I

MOLECULAR CLONING, SEQUENCE ANALYSIS AND DEVELOPMENTAL EXPRESSION OF CAROTENOID BIOSYNTHETIC GENES IN *Citrus paradisi* (Macf.)

ABSTRACT 6

INTRODUCTION 7

MATERIALS AND METHODS 12

Plant materials 12

Isolation and sequence analysis of the citrus carotenoid biosynthetic genes .. 12

Relative quantitative RT-PCR 13

RESULTS 16

Isolation of the carotenoid biosynthetic genes from grapefruit 16
Sequence analysis of the isolated carotenoid biosynthetic genes from different grapefruit cultivars 17
Expression analysis of the isolated carotenoid biosynthetic genes during grapefruit fruit development 26
DISCUSSION 30
REFERENCES 37

CAPÍTULO II

AN EXAMINATION OF THE MORPHOGENIC GRADIENTS OF ADVENTITIOUS BUD AND SHOOT REGENERATION IN EPICOTYL EXPLANTS OF CITRUS

ABSTRACT 43
INTRODUCTION 44
MATERIALS AND METHODS 47
Plant materials 47
Culture medium and conditions of incubation 47
Experimental design and statistical analysis 50
RESULTS 51
In vitro morphogenic responses in epicotyl segments to different concentrations of BAP 51
Effect of BAP concentration on the gradients of bud and shoot regeneration 52
Effect of light regime on the gradients of bud and shoot regeneration 54
Effect of age of the seedling on the gradients of bud and shoot regeneration 59
DISCUSSION 62
REFERENCES 67

CAPÍTULO III

AN EVALUATION OF FACTORS AFFECTING THE EFFICIENCY OF *Agrobacterium*-MEDIATED TRANSFORMATION OF *Citrus paradisi* (Macf.) AND OBTENTION OF TRANSGENIC PLANTS CONTAINING CAROTENOID BIOSYNTHETIC GENES UNDER CONSTITUTIVE EXPRESSION

ABSTRACT 70

INTRODUCTION 71

MATERIALS AND METHODS 73

Plant material and *Agrobacterium* transformation 73

Agrobacterium strains and plasmids 73

Factors evaluated 74

Hygromycin as a selective agent 74

BiBAC as a transformation vector 74

Experimental design 75

Polymerase chain reaction (PCR) analysis 75

Southern hybridization 76

RESULTS AND DISCUSSION 78

Analysis of the factors affecting the transformation efficiency of 'Duncan' grapefruit 78

Preculturing 78

Incubation period 79

Agrobacterium concentration 80

Cocultivation medium 80

Cocultivation period 81

BAP concentration in selective regeneration medium 82

Improved protocol 84

Incorporation of carotenoid biosynthetic genes under constitutive expression 86

Hygromycin as a selective agent in citrus 86
BiBAC as a transformation vector in citrus 89
REFERENCES 91
CONCLUSÕES GERAIS 95

RESUMO

COSTA, Marcio Gilberto Cardoso, D.S., Universidade Federal de Viçosa, junho de 2002. **Morfogênese *in vitro*, transformação genética, clonagem e superexpressão de genes da rota biossintética de carotenóides em citros.** Orientador: Wagner Campos Otoni. Conselheiros: Luiz Orlando de Oliveira, Paulo Roberto Mosquim e Sérgio Hermínio Brommonschenkel.

Há muito tempo que as características relacionadas à coloração do fruto e sua qualidade nutricional têm sido consideradas desejáveis para manipulação em citros. Entretanto, a produção de novas cultivares dessas plantas tem sido limitada pelos vários obstáculos que impedem o seu melhoramento genético. A biotecnologia, por meio das técnicas de cultura de tecidos e transformação genética de plantas, surge como uma opção viável para contornar esse problema. Os objetivos do presente estudo foram isolar e caracterizar alguns genes envolvidos na rota biossintética de carotenóides de *Citrus paradisi* (Macf.) e desenvolver metodologias de regeneração *in vitro* e transformação genética para manipulação dessa rota biossintética em citros, visando alterar o conteúdo de provitamina *A*, a coloração do fruto e o porte das plantas. Utilizando-se a técnica de RT-PCR, as seqüências de cDNAs dos genes da sintase do fitoeno (AF152892), desaturase do fitoeno (AF364515), desaturase do ζ-caroteno (AF372617), ciclase-β do licopeno (AF152246) e ciclase-ε do licopeno (AF486650) foram isoladas de *C. paradisi*. Em geral, essas seqüências apresentaram elevada homologia aos genes correspondentes em tomate, com

identidade de 72-83% em nível de aminoácidos. Dois ou mais transcritos diferentes foram identificados para três dos cinco genes caracterizados nesse estudo. Em cada caso, um ou mais transcritos foram considerados aberrantes, em que a produção de polipeptídeos não-funcionais foi predita. Estudos de expressão gênica revelaram que a maioria dos genes isolados é transcricionalmente regulada durante o desenvolvimento do fruto. No entanto, a diferenciação da coloração do fruto entre as cultivares de *C. paradisi* pode ser causada por mutações na seqüência aberta de leitura e não por regulação transcricional diferencial, sendo a desaturase do fitoeno e/ou ciclase-ε do licopeno os genes candidatos pelas diferenças observadas. Na avaliação da morfogênese *in vitro* de tecidos derivados de epicótilos de limão 'Cravo' (*C. limonia* Osb.), pomelo 'Foster' (*C. paradisi* Macf.) e laranja 'Pêra' [*C. sinensis* (L.) Osb.], verificou-se diferentes respostas às condições de cultivo *in vitro* em função da cultivar, da região do epicótilo utilizada como fonte de explantes, da composição do meio de cultura e das condições de incubação. Um sistema eficiente de transformação genética via *Agrobacterium tumefaciens* foi desenvolvido para *C. paradisi*, examinando-se os efeitos de seis fatores na eficiência de transformação. A pré-cultura dos explantes e a composição do meio de co-cultivo foram os fatores que mais influenciaram a eficiência de transformação. O protocolo otimizado foi empregado na produção de plantas transgênicas contendo os genes da sintase do fitoeno, desaturase do fitoeno ou ciclase-β do licopeno sob expressão constitutiva.

ABSTRACT

COSTA, Marcio Gilberto Cardoso, D.S., Universidade Federal de Viçosa, June 2002. ***In vitro*** **morphogenesis, genetic transformation, cloning and overexpression of carotenoid biosynthetic genes in citrus.** Adviser: Wagner Campos Otoni. Committee members: Luiz Orlando de Oliveira, Paulo Roberto Mosquim and Sérgio Hermínio Brommonschenkel.

Fruit color and its nutritional value have been considered desirable for manipulation in citrus for a long time. However, citrus breeding has been limited due to several factors that hinder its genetic improvement. Plant biotechnology appears to be a viable option for the improvement of citrus species by means of the plant tissue culture and genetic transformation techniques. The objectives of this study were to isolate and characterize some genes involved in the carotenoid biosynthetic pathway of *Citrus paradisi* (Macf.) and to develop protocols of *in vitro* regeneration and genetic transformation for manipulation of this pathway in citrus, aiming to change the provitamin A content, fruit color, and plant height. By using the RT-PCR technique, the cDNA sequences of the genes phytoene synthase (AF152892), phytoene desaturase (AF364515), ζ-carotene desaturase (AF372617), lycopene β-cyclase (AF152246), and lycopene ε-cyclase (AF486650) were isolated from *C. paradisi*. In general, they were highly homologous to the corresponding tomato genes at the amino acid level, with identity ranging from 72-83%. Two or more different transcripts were identifyed for three of the five genes characterized in this study. In each case, one or more

of the transcripts were aberrant, so that the production of a nonfunctional protein would be predicted. The expression analysis of the isolated carotenoid biosynthetic genes indicated complex expression patterns during fruit development. However, the fruit color differentiation between the grapefruit cultivars may be caused by frame-shift mutation and not by differential transcriptional regulation, with phytoene desaturase and/or lycopene ε-cyclase being the candidate genes for fruit color differences. The *in vitro* responses of epicotyl explants from 'Cravo' rangpur lime (*Citrus limonia* Osb.), 'Foster' grapefruit (*C. paradisi* Macf.), and 'Pêra' sweet-orange [*C. sinensis* (L.) Osb.] varyed according to cultivar, region of the epicotyl used as source of explant, culture medium composition, and incubation conditions. An improved protocol for *Agrobacterium*-mediated transformation of epicotyl explants from 'Duncan' grapefruit was developed by examining the effects of six different factors on the efficiency of transformation and combining the best treatments for each factor. The preculturing of the explants and the composition of the cocultivation medium were the factors that most influenced transformation efficiency. The optimized protocol was successfully employed in the production of transgenic grapefruit plants containing the carotenoid biosynthetic genes phytoene synthase, phytoene desaturase, or lycopene β-cyclase under constitutive expression.

INTRODUÇÃO GERAL

Os citros têm sido reconhecidos como um dos grupos de plantas economicamente mais importantes do mundo. Frutas cítricas são produzidas comercialmente em 90 países, sendo o Brasil o maior produtor mundial e exportador de suco de laranja concentrado. Cerca de 70% da produção mundial de citros é representada pela laranja-doce (*Citrus sinensis*), com o Brasil respondendo por 30% desse total. Dentre as cultivares de laranja-doce, a 'Pêra' é a mais importante da citricultura brasileira, sendo responsável por 45% da produção total de laranjas-doce. Os seus frutos são os preferidos e os mais procurados para a fabricação de suco concentrado para a exportação, além de terem excelente aceitação nos mercados internos e externos de frutas *in natura*.

Em plantas cítricas, a coloração do fruto é devida principalmente aos carotenóides, responsáveis pelas típicas colorações amarela, laranja e vermelha. Em adição aos hidrocarbonos fitoeno e licopeno, quatro principais xantofilas contribuem para a coloração do fruto: β-citraurina, criptoxantina, violaxantina e anteraxantina (Stewart e Wheaton, 1973). O modelo de distribuição de carotenóides no fruto é específico de cada espécie, e dentro das espécies, a distribuição de carotenóides pode variar de acordo com a cultivar, ambiente, maturidade do fruto, condições de crescimento e variações sazonais (Gross, 1977).

O conhecimento da seqüência de reações bioquímicas que constituem a rota biossintética de carotenóides em plantas e a disponibilidade dos genes clonados codificando para a maioria das enzimas, tornam essa rota biossintética atrativa para a engenharia metabólica. O interesse na manipulação do conteúdo e da composição de carotenóides em plantas tem sido cada vez mais crescente (Hirschberg, 2001). Todas as espécies de carotenóides que contêm o anel *b* podem ser convertidas a retinol e, portanto, são precursoras de vitamina *A*. Embora esse seja o principal valor de carotenóides na nutrição humana, benefícios adicionais à saúde são atribuídos à sua atividade antioxidante *in vivo*, que ajuda a reduzir o risco de doenças coronárias e o desenvolvimento de certas formas de câncer (Mayne, 1996). Aplicações industriais de carotenóides incluem seu uso como produtos farmacêuticos, cosméticos e corantes em alimentos. Como pigmentos naturais, carotenóides fornecem colorações atrativas a frutos e flores ornamentais, e sua composição nessas plantas tem um importante valor econômico.

Pouco sucesso na manipulação genética de citros tem sido obtido em virtude dos vários obstáculos que impedem o seu melhoramento, incluindo a esterilidade parcial ou completa do pólen e óvulo, auto-incompatibilidade e incompatibilidade cruzada, apomixia, longo período de juvenilidade, elevada heterozigose e desconhecimento da natureza genética e modo de herança da maioria das características de importância agronômica (Gmitter et al., 1992). A biotecnologia, por meio das técnicas de cultura de tecidos e transformação genética de plantas, surge como uma opção viável para contornar esse problema. O método mais utilizado na transformação genética de citros é a transformação mediada por *Agrobacterium*, utilizando-se segmentos de epicótilos como explantes (Moore et al., 1992; Kaneyoshi et al., 1994; Peña et al., 1995; Peña et al., 1997; Gutiérrez-E. et al., 1997; Bond e Roose, 1998; Cervera et al., 1998; Luth e Moore, 1999). Apesar da disponibilidade de protocolos de transformação genética para diversas espécies de citros, a eficiência de transformação é relativamente baixa quando comparada a plantas de outras espécies. Os

principais problemas têm sido o baixo número de plantas regeneradas, a dificuldade de enraizamento e o elevado número de escapes.

Os objetivos do presente estudo foram identificar, isolar e caracterizar a seqüência de cDNA dos genes envolvidos nas primeiras reações da rota biossintética de carotenóides de *Citrus paradisi* (Macf.), e desenvolver metodologias de regeneração *in vitro* e transformação genética visando a manipulação genética dessa rota biossintética em citros. Na primeira parte do estudo, iniciadores derivados de seqüências conservadas dos genes da rota biossintética de carotenóides de arabidopsis, tomate, tabaco, pimentão, soja e/ou melão foram usados para a clonagem de seqüências homólogas em *C. paradisi* via RT-PCR. Estudos de expressão gênica durante o desenvolvimento do fruto foram efetuados visando caracterizar a expressão dos genes isolados em diferentes tecidos (albedo e vesículas de suco/parede carpelar) de duas cultivares de *C. paradisi* (‘Duncan’ e ‘Flame’). Na segunda parte do estudo, a morfogênese *in vitro* em segmentos de epicótilos de limão ‘Cravo’ (*C. limonia* Osb.), pomelo ‘Foster’ (*C. paradisi* Macf.) e laranja ‘Pêra’ [*C. sinensis* (L.) Osb.] foi avaliada. Investigações adicionais foram efetuadas em limão ‘Cravo’ e pomelo ‘Foster’ visando-se caracterizar a resposta morfogênica de cinco regiões do epicótilo sob diferentes tratamentos. Finalmente, um sistema eficiente de transformação genética via *Agrobacterium tumefaciens* foi desenvolvido para *C. paradisi*, examinando-se os efeitos de seis fatores na eficiência de transformação. O protocolo otimizado foi empregado na produção de plantas transgênicas contendo os genes da rota biossintética de carotenóides sintase do fitoeno, desaturase do fitoeno ou ciclase-β do licopeno sob expressão constitutiva.

REFERÊNCIAS BIBLIOGRÁFICAS

BOND, J.E.; ROOSE, M.L. *Agrobacterium*-mediated transformation of the commercially important citrus cultivar Washington navel orange. **Plant Cell Reports**, v. 18, n. 3-4, p. 229-234, 1998.

CERVERA, M.; PINA, J.A.; JUAREZ, J.; NAVARRO, L.; PEÑA, L. *Agrobacterium*-mediated transformation of citrange: factors affecting transformation and regeneration. **Plant Cell Reports**, v. 18, n. 3-4, p. 271-278, 1998.

GMITTER, Jr., F.G.; GROSSER, J.W.; MOORE, G.A. Citrus. In: HAMMERSCHLAG, E.F.; LITZ, R.E. (Eds.). **Biotechnology of perennial fruit crops.** Cambridge: CAB International, 1992. p. 335-369.

GROSS, J. Carotenoid pigments in citrus. In: NAGY, S; SHAW, P.E.; VELDHUIS, M. (Eds.). **Citrus Science and Technology.** Westport: Avi. Pub. Co., 1977. v. 1, p. 302-354.

GUTIÉRREZ-E, M.A.; LUTH, D.; MOORE, G.A. Factors affecting *Agrobacterium*-mediated transformation in *Citrus* and production of sour orange (*Citrus aurantium* L.) plants expressing the coat protein gene of citrus tristeza virus. **Plant Cell Reports**, v. 16, n. 11, p. 745-753, 1997.

HIRSCHBERG, J. Carotenoid biosynthesis in flowering plants. **Current Opinion in Plant Biology**, v. 4, n. 3, p. 210-218, 2001.

KANEYOSHI, J.; KOBAYASHI, S.; NAKAMURA, Y.; SHIGEMOTO, N.; DOI, Y. A simple and efficient gene transfer system of trifoliate orange (*Poncirus trifoliata* Raf). **Plant Cell Reports**, v. 13, n. 10, p. 541-545, 1994.

LUTH, D.; MOORE, G.A. Transgenic grapefruit plants obtained by *Agrobacterium tumefaciens*-mediated transformation. **Plant Cell Tissue and Organ Culture**, v. 57, n. 3, p. 219-222, 1999.

MAYNE, S.T. Beta-carotene, carotenoids, and disease prevention in humans. **FASEB Journal**, v. 10, n. 7, p. 690-701, 1996.

MOORE, G.A.; JACONO, C.C.; NEIDIGH, J.L.; LAWRENCE, S.D.; CLINE, K. *Agrobacterium*-mediated transformation of *Citrus* stem segments and regeneration of transgenic plants. **Plant Cell Reports**, v. 11, n. 5-6, p. 238-242, 1992.

PEÑA, L.; CERVERA, M.; NAVARRO, A.; PINA, J.A.; DURÁN-VILA, N.; NAVARRO, L. *Agrobacterium*-mediated transformation of sweet orange and regeneration of transgenic plants. **Plant Cell Reports**, v. 14, n. 10, p. 616-619, 1995.

PEÑA, L.; CERVERA, M.; JUAREZ, J.; NAVARRO, A.; PINA, J.A.; NAVARRO, L. Genetic transformation of lime (*Citrus aurantifolia* Swing): factors affecting transformation and regeneration. **Plant Cell Reports**, v. 16, n. 11, p. 731-737, 1997.

STEWART, I.; WHEATON, T.A. Carotenoids in citrus. **Florida Agricultural Experimental Stations Journal Series**, n. 4887, p. 325-330, 1973.

CAPÍTULO I

MOLECULAR CLONING, SEQUENCE ANALYSIS AND DEVELOPMENTAL EXPRESSION OF CAROTENOID BIOSYNTHETIC GENES IN *Citrus paradisi* (Macf.)

ABSTRACT

In *Citrus paradisi* (Macf.), the fruit can be either red or white due to a family of plant pigments known as carotenoids. The tools of molecular biology were used to elucidate the genetic basis of the fruit color differentiation between the grapefruit cultivars. We have isolated the cDNA sequence of the genes phytoene synthase, phytoene desaturase, ζ-carotene desaturase, lycopene β-cyclase, and lycopene ε-cyclase, involved in the early steps of the carotenoid biosynthetic pathway from *C. paradisi*, and then performed sequence analysis and expression studies as an attempt to understand the process that causes color differentiation to occur. The presence of aberrant phytoene desaturase and lycopene ε-cyclase transcripts suggests one or both of them as the candidate gene(s) for fruit color differences. In addition, other important aspects about the molecular biology of the carotenoid biosynthetic pathway in *C. paradisi* are discussed based in our data.

Key words: carotenoid, gene expression, grapefruit, *Citrus paradisi*

INTRODUCTION

Carotenoids are a large family of isoprenoid pigments that are essential components in light harvesting systems and in photosynthetic reaction centers of all photosynthetic organisms (Britton, 1995; Niyogy, 1999). Carotenoids can be further metabolized to abscisic acid in plants (Zeevaart and Creelman, 1988) and to vitamin A in animals (Mayne, 1996). An additional role of carotenoids in higher plants is as coloring agents in flowers and fruits to attract pollinators and agents of seed dispersal (Goodwin, 1980). In these tissues, the carotenoids accumulate in nonphotosynthetic chromoplasts via the general isoprenoid biosynthetic pathway (Figure 1). The types of carotenoid(s) that accumulate range widely between species from intermediates in the pathway, such as lycopene in tomatoes, to species-specific ones such as in pepper fruits. All enzymes involved in the carotenoid pathway are nuclear encoded and their polypeptide products are imported to the plastids. Most of the genes encoding these enzymes have been cloned in recent years (reviewed by Cunningham and Gantt, 1998).

The process of carotenoid accumulation in chromoplasts has been extensively studied in ripening tomato fruits because of the dramatic color changes that occur during this process and also the availability of a large collection of color mutants (reviewed by Hirschberg, 2001). At the ‘breaker’ stage of ripening, the fruit color begins to change to red because of the accumulation of lycopene. It has been established that differential transcriptional

regulation plays an important role in this process. The mRNA levels for the enzymes that produce lycopene, phytoene synthase (PSY), phytoene desaturase (PDS), carotenoid isomerase (CRTISO), and plastid terminal oxidase (PTOX), increase 4-20-fold at this stage, and at the same time, the mRNAs of both lycopene cyclases disappear (Pecker et al, 1996; Ronen et al., 1999; Josse et al., 2000; Isaacson et al., 2002; Park et al., 2002). Transcriptional upregulation of carotenoid biosynthesis genes also appears to be the major regulatory mechanism in carotenogenesis that takes place in flowers of daffodil (Schledz et al., 1996) and marigold (Moehs et al., 2001). In developing pepper fruit, on the other hand, while expression of some carotenoid biosynthetic enzymes increases with ripening, other enzymes are lowly or constitutively expressed (Hugueney et al., 1992,1995; Josse et al., 2000).

Carotenoids are responsible for the characteristic yellow, orange, and red colorations of mature citrus fruit. Unusually, β-citraurin, a C-30 carotenoid, is in part responsible for the orange and red color of mandarins and oranges (Stewart, 1977). C-40 carotenoids are also present, however cryptoxanthin (orange) and violaxanthin and antheraxanthin (yellow) and their esters are the primary xanthophylls present in most citrus fruits; the hydrocarbon carotenoids, such as phytoene and lycopene, are usually present in low concentrations (Curl and Bailey, 1956; Stewart and Wheaton, 1973; Stewart, 1977; Philip et al., 1988; Oberholster et al., 2001). Carotenoid composition in the peel of the fruit (consisting of the spongy, usually white albedo and narrow flavedo layer on the exterior that provides peel color) typically differs both quantitatively and qualitatively from that of the endocarp (juice vesicles and segment wall tissue) of the fruit (Gross, 1977). Carotenoid increase is not linear in maturing citrus fruit; levels may suddenly rise 4-fold in the fruit interior and 9-fold in peel at color break, when chlorophyll has mostly disappeared and color in the fruit is increasing (Gross, 1977).

The situation with grapefruit (*C. paradisi* Macf.) is unique from that of other citrus species in several respects. First, the origin of the type (it is a hybrid, not a true species) is known as to time, place and parentage; a seedy grapefruit

hybrid was produced from a cross between a pummelo [*Citrus grandis* (L.) Osb.] and a sweet orange (*C. sinensis* Osb.) in the eighteenth century in the West Indies (Gmitter, 1995; Moore, 2001). The origins of most citrus types are unknown. Secondly, the carotenoid composition of grapefruit differs from most citrus fruits. Carotenoids in the pale ivory-yellow colored fruit of the grapefruit type that has been termed white consist mainly of uncolored hydrocarbons phytoene (50%) and phytofluene (24%); pink and red grapefruit also accumulate the red-colored hydrocarbon lycopene and β-carotene (Gross, 1977; Purcell et al., 1961; Yokoyama and White, 1967). Thus, the mixture of the colored carotenoids is less complex than in other citrus fruits. Third, the origins of all grapefruit cultivars grown today are known; the time and place where most varieties of other citrus types were selected or bred were not recorded. A white seedy grapefruit type was imported into Florida in the early nineteenth century. Successive somatic mutations conferring seedlessness and pink-fleshed and subsequently red-fleshed fruit were identified and vegetatively propagated in Florida and Texas (Cameron et al., 1964) (Figure 2).

Much has been written in the literature about carotenoid types and levels in these different varieties of grapefruit, but little is known about the molecular biology of the carotenoid biosynthetic pathway in *C. paradisi* and what process is involved in the observed color differentiation between the cultivars. In this study, we describe the molecular cloning, sequence analysis and developmental expression of the genes involved in the early steps of the carotenoid biosynthetic pathway from *C. paradisi*, as the first attempt in a comprehensive analysis of the process that causes color differentiation to occur in its fruit.

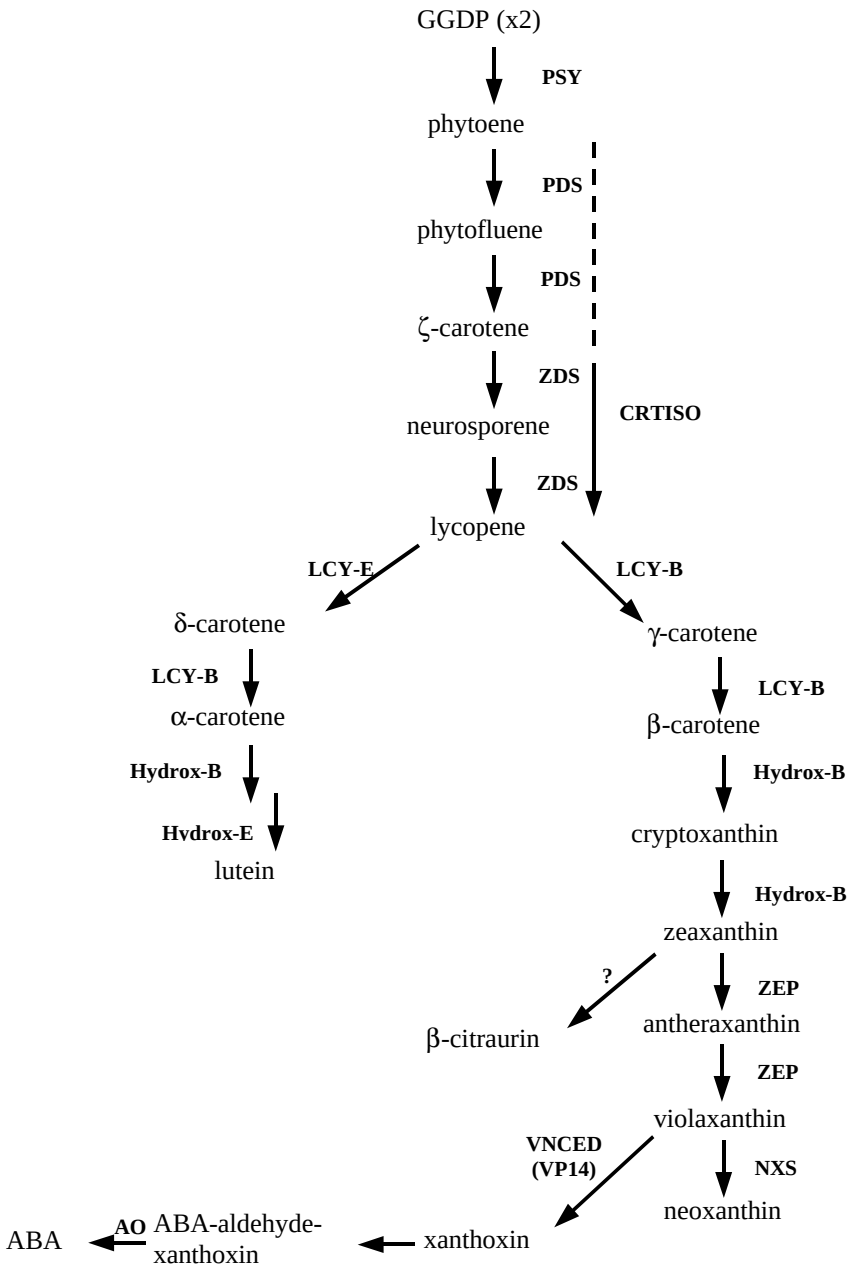


Figure 1- The carotenoid biosynthetic pathway in plants. Enzymes are abbreviated as follows: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LCY-E, lycopene-ε-cyclase; LCY-B, lycopene-β-cyclase; Hydrox-E, ε-ring hydroxylase; Hydrox-B, β-ring hydroxylase; ZEP, zeaxanthin epoxidase; NXS, neoxanthin synthase; VNCED (VP14), 9-cis-epoxycarotenoid dioxygenase; AO, aldehyde oxidase. GGDP, geranylgeranyl diphosphate; ABA, abscisic acid.

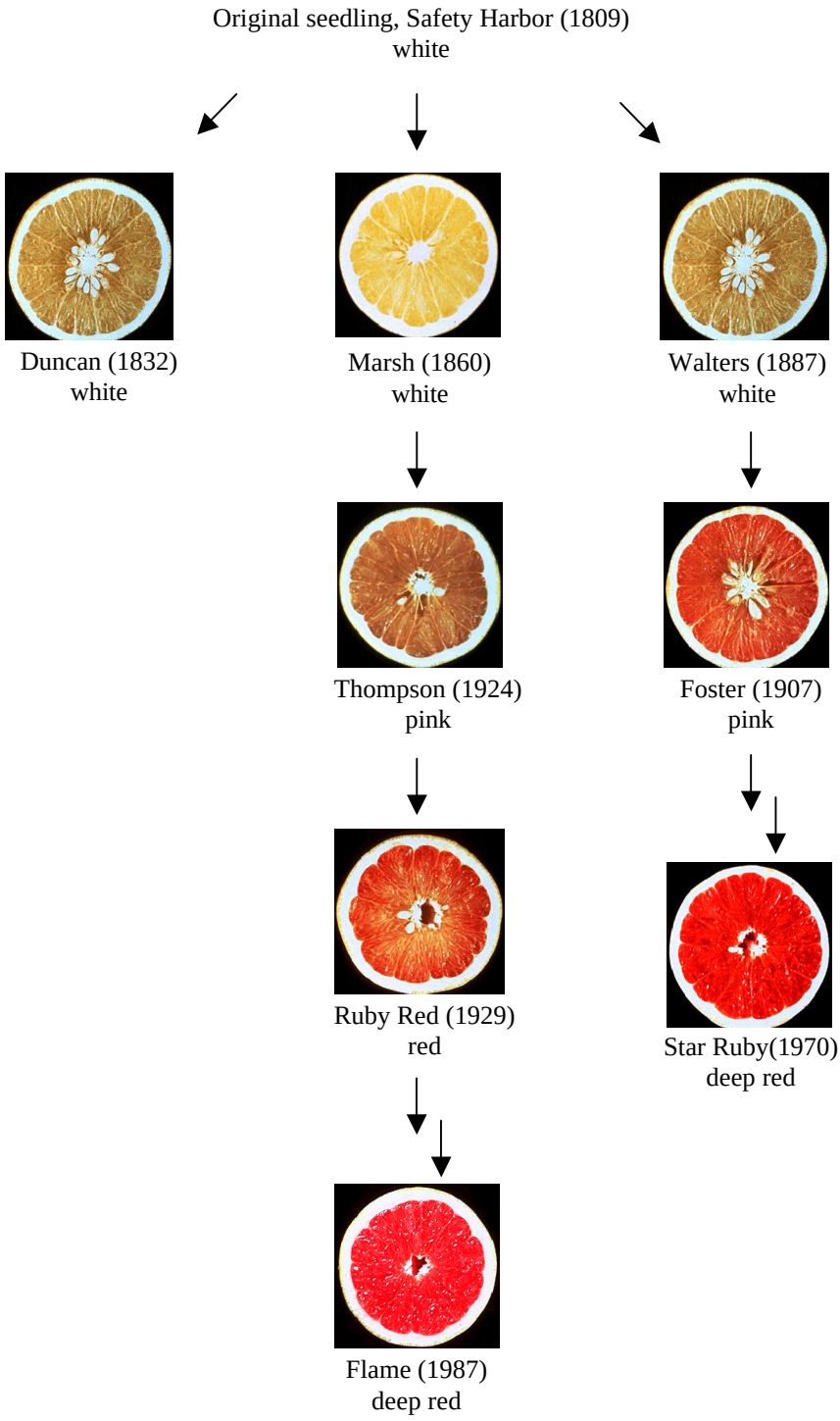


Figure 2- Origin of some of the most important grapefruit cultivars in Florida and Texas. Number in parentheses indicates date of discovery or release.

MATERIALS AND METHODS

Plant materials

Fruit samples from different grapefruit (*Citrus paradisi* Macf.) cultivars were collected at the Citrus Research and Education Center (CREC), Lake Alfred, FL. The cultivars included 'Duncan' (white flesh, white peel), 'Thompson' (pink flesh, white peel), 'Ruby Red' (red flesh, red on peel), 'Star Ruby' (deep red flesh, red on peel), and 'Flame' (deep red flesh, red on peel). Albedo (internal layer of the peel) and juice vesicles/segment wall tissue (endocarp) were separated from each other, cut in pieces and frozen immediately in liquid nitrogen after wrapping in aluminium foil. The fruit samples were kept at -80^{o}C until use.

Isolation and sequence analysis of the citrus carotenoid biosynthetic genes

Total RNA was isolated from albedo and juice vesicle tissue from the grapefruit cultivars using the protocol described by Chang et al. (1993). Reverse transcription was carried out using the RETROscript kit (Ambion Inc., Austin, USA), following the manufacturer's instructions. For isolation of the phytoene synthase, phytoene desaturase, ζ-carotene desaturase, lycopene β-cyclase, and lycopene ε-cyclase cDNA fragments, sense and antisense primers were synthesized based on conserved regions of the respective carotenoid biosynthetic genes from arabidopsis, tomato, tobacco, pepper, soybean and/or melon,

published in the GenBank Database at National Center for Biotechnology Information (NCBI). The amplified cDNA fragments were cloned into pGEM-T vector using the TA cloning system (Promega Co., Madison, USA). *E. coli* strain XL1-Blue (Stratagene, La Jolla, USA) was used this experiment and the ones below. After confirmation of the identity of the amplified products as the respective carotenoid biosynthetic genes by DNA sequencing, new primers were synthesized based on these sequences and employed in reactions of rapid amplification of cDNA ends (RACE), using the SMART^{TM} RACE cDNA Amplification kit (Clontech Laboratories Inc., Palo Alto, USA). After characterization of the RACE products by sequencing, full-length cDNA copies of the carotenoid biosynthetic genes from *C. paradisi* were generated by long distance PCR (LD PCR), using primers designed from the extreme 5' and 3' ends of the cDNAs.

Relative quantitative RT-PCR

The relative abundance of the phytoene synthase, phytoene desaturase, ζ-carotene desaturase and lycopene β-cyclase mRNAs in samples of albedo and juice vesicle/segment wall tissue from fruits of 'Duncan' and 'Flame' grapefruit cultivars was assessed employing the QuantumRNA 18S Internal Standards kit (Ambion Inc., Austin, USA). Samples of the fruit tissues from both cultivars were collected throughout the time course of fruit development, as follows: 05/28/98: fruits with 4-5 cm diameter, both albedo and juice vesicles were white in 'Flame'; 06/11/98: fruits with 5-6 cm diameter, albedo pink and juice vesicles white in 'Flame'; 06/25/98: fruits with 6-7 cm diameter, albedo pink and juice vesicles faint pink in 'Flame'; 07/14/98: fruits with 7-8 cm diameter, both albedo and juice vesicles pink in 'Flame'; 08/03/98: fruits with 8 cm diameter, albedo pink and juice vesicles red in 'Flame'; 08/27/98: fruits with 8.5 cm diameter, both albedo and juice vesicles red in 'Flame'; 09/16/98: fruits with 8.5 cm diameter, albedo red and juice vesicles deep red in 'Flame'; 10/09/98: fruits with 9 cm diameter, albedo pink and juice vesicles deep red in 'Flame'; 11/04/98: fruits with 9.5 cm diameter, albedo faint pink and juice vesicles red in 'Flame';

12/04/98: fruits with 10 cm diameter, albedo faint pink and juice vesicles faint red in 'Flame'; 02/10/99: fruits with 10 cm diameter, albedo faint pink and juice vesicles faint red in 'Flame'.

Prior to RT-PCR reactions, total RNA was treated with DNase I (FPLC pure, Amersham Pharmacia Biotech) to avoid DNA contamination (100 U Dnase I per μg total RNA). Reverse transcription was carried out using 2 μg of total RNA and random hexamers as primers, according to manufacturer's instructions. The amplification procedure by PCR consisted of 1 min at 93^{o}C, 1 min at 60 ^{o}C, and 3 min at 72 ^{o}C, 35 cycles, as determined by the linear range analysis of the genes. The optimal ratio of 18S primers:competimers determined was 2:8, characteristic of rare transcripts. For the purpose of quantification, the dNTP mix of the PCR reactions was replaced by PCR DIG Labeling Mix (Roche Molecular Biochemicals, Mannheim, Germany), which incorporates digoxigenin-11-dUTP into the PCR products.

The following primers were used for the PCR amplification: for phytoene synthase, 5'-GCTTCGGGTTCGTCGATTCAGTTC-3' (forward) and 5'-ATAG-GGCATCGGCCTTGGATTGTG-3' (reverse); for phytoene desaturase, 5'-CCA-AACCTTCTCTCTGCTGAGTTC-3' (forward) and 5'-GCTCCAATTATAAA-CCCTGCCTCC-3' (reverse); for ζ-carotene desaturase, 5'-CTCTCTAAAACA-GTTCAGTTCCCTG-3' (forward) and 5'-CATAGGTATTGGAAACCCTTAC-TCC-3' (reverse); for lycopene β-cyclase, 5'-CAGGCCACAAACGCAACACA-AGC-3' (forward) and 5'-CCATCCCAG-CGGTACCACCTATTC-3' (reverse).

Products of the PCR amplification were separated by electrophoresis in 2% agarose gels and then transferred to nylon membranes (Boehringer Mannheim, Mannheim, Germany). The membranes were taken through the DIG chemiluminescent detection procedure as recommended by the manufacturer (Roche). The products of the PCR were quantified using the program AlphaEase V5.5. In each sample, the signal obtained for the gene specific amplicon was divided by the signal obtained for the 18S amplicon. This yielded a corrected relative value for the gene specific product in each sample. These values were

compared between samples for an estimate of the relative expression of target RNA in the samples.

RESULTS

Isolation of the carotenoid biosynthetic genes from grapefruit

To isolate grapefruit cDNA sequences encoding the genes from the carotenoid biosynthetic pathway, we first searched the sequences of the genes of interest from various plant species available in the databases of the National Center for Biotechnology Information (NCBI). The sequences were compared for highly conserved regions on both peptide and nucleotide levels and primers were then synthesized and used in PCR reactions performed on first strand cDNA derived from reverse transcription (RT) of *C. paradisi* fruit mRNA. Several fragments of interest of the approximate predicted sizes of the carotenoid biosynthetic transcripts were cloned and sequenced. The sequences were compared to known cDNAs encoding carotenoid biosynthetic enzymes using the BLAST algorithm (Altschul et al., 1990) at the NCBI. After the successful identification of the amplified products as carotenoid biosynthetic transcripts, full-length cDNAs were generated employing the system of rapid amplification of cDNA ends (RACE).

The genes in the carotenoid pathway that we have cloned from grapefruit are listed in Table 1. In general, they were highly homologous to the corresponding tomato genes at the amino acid level, with the identity ranging from 72% to 83%. The identity is higher when the less conserved N-terminal putative plastid targeting signals are not considered. Analysis of the translation of the genes described with the chloroplast targeting signal recognition program

ChloroP (Emanuelsson et al., 1999) indicated that each cDNA contained a high-confidence plastid targeting signal.

Table 1- Information about the carotenoid biosynthetic genes discussed in this report including the acession number. Abbreviations: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LCY-B, lycopene β-cyclase; LCY-E, lycopene ε-cyclase.

cDNA	Nucleotides	Amino acids	% amino acid identity with tomato homologue	Acession number
PSY	1773	436	72	AF152892
PDS	1944	552	83	AF364515
ZDS	2245	570	83	AF372617
LCY-B	1934	504	80	AF152246
LCY-E	1550	437	75	AF486650

Sequence analysis of the isolated carotenoid biosynthetic genes from different grapefruit cultivars

Since the fruit color differentiation between the grapefruit cultivars could be attributed to changes in the open reading frame (ORF) of the genes, the cDNA sequences of the isolated carotenoid biosynthetic genes were compared between the different grapefruit cultivars. Total RNA was isolated from albedo and juice vesicles/segment wall tissue of ‘Duncan’, ‘Thompson’, ‘Ruby Red’, ‘Star Ruby’, and ‘Flame’, and used in RT-PCR reactions. The amplified cDNA fragments of each gene were cloned and sequenced. Sequence analysis was then performed aiming to identify gene differences at the nucleotide level between the cultivars. Surprisingly, the grapefruit cultivars contained aberrant forms of the isolated genes in addition to normal transcripts for three of the five genes analyzed. A normal, or wild-type, transcript was one that had homology to previously cloned genes and was predicted to encode a functional protein (see discussion), while an aberrant transcript was one that was predicted to encode a nonfunctional transcript. Phytoene synthase and ζ-carotene desaturase genes revealed only

normal transcripts from any cultivar or tissue. Comparison of the lycopene β-cyclase cDNA sequence between the different grapefruit cultivars revealed the presence in all of them of a transcript with a 104 bp deletion along with normal transcripts, irrespective of the fruit tissue analyzed (albedo or juice vesicles) (Figure 3). This deletion, which is located 28 bp before the beginning of the ORF, has apparently created a new start codon in the aberrant transcripts that begins before the regular one, adding 20 new amino acids to the N-terminal end of the lycopene β-cyclase enzyme (Figure 4). As the N-terminal end of the proteins imported to plastids is involved in targeting, it is likely that the new amino acids added to the N-terminal end of the aberrant lycopene β-cyclase enzyme have disrupted its chloroplast transit peptide (cTP), preventing its import to plastids. Indeed, a plastid targeting signal was not recognized in the aberrant transcript using the chloroplast signal recognition program ChloroP (Emanuelsson et al., 1999) (results not shown).

Analysis of the lycopene ε-cyclase cDNA sequence showed initially that all grapefruit cultivars evaluated have four different transcripts from this gene in both albedo and juice vesicles/segment wall tissue. One transcript is 1550 bp long and encodes a normal protein of 437 amino acids, as characterized in Table 1. The other transcripts are 1584 (mutant 1), 1657 (mutant 2), and 1570 (mutant 3) bp long and contain a disrupted ORF caused by insertions and deletions in the 5' end of the lycopene ε-cyclase gene (Figure 5). A polypeptide containing only the last 298 amino acids would be predicted in two aberrant transcripts (mutant 1 and mutant 2). The genetic alterations present in the third aberrant transcript (mutant 3) have produced a new ORF encoding a shorter lycopene ε-cyclase enzyme (424 amino acids), containing 12 different amino acid residues in its N-terminal end. A plastid targeting signal was not identifyed in this transcript.

A more detailed analysis of the "normal" lycopene ε-cyclase transcripts has revealed subtle differences of sequence in the different grapefruit cultivars (Figure 6). Comparing lycopene ε-cyclase transcripts from 'Duncan' and 'Thompson', differences in the nucleotides 621, 708, and 1227 change the amino acid residues at positions 193, 222, and 395 of the polypeptide (Figure 6a).

Differences in six nucleotides were also found in a lycopene ε-cyclase transcript from Flame, changing the amino acid residues at six positions of the polypeptide (Figure 6b). The difference in the nucleotide 1356 has a more drastic consequence, since it changes the stop codon of the polypeptide, producing a predicted product containing 20 additional amino acid residues in its C-terminal end. The sequence similarity (>99%) between lycopene ε-cyclase transcripts of 'Duncan' and those found in 'Thompson' and 'Flame' does not permit us to distinguish in hybridization experiments whether 'Duncan' also contains those transcripts or not. However, the same transcript found in 'Duncan' was also identifyed in 'Thompson' and 'Ruby Red', indicating that different isoforms of lycopene ε-cyclase may exist in citrus. An important clue indicating that the lycopene ε-cyclase gene may be involved in fruit color differentiation came from the sequence of the transcripts found in 'Ruby Red' and 'Star Ruby'. A single-base deletion of T in nucleotide 227 of a lycopene ε-cyclase transcript found in 'Ruby Red' causes a frame-shift after the Glu-60 codon that produces a truncated polypeptide of 87 amino acids (Figure 6c). The same situation was observed in a lycopene ε-cyclase transcript from 'Star Ruby', in which a single-base deletion of T in nucleotide 619 produces a frame-shift after the Val-191 codon, and a truncated polypeptide containing only 241 amino acids is produced (Figure 6d).

A phytoene desaturase transcript containing a 22 bp deletion and a 46 bp insertion inside the ORF was identified in 'Duncan' grapefruit in addition to normal transcripts (Figure 7). This aberrant form has putatively disrupted the PDS ORF by introducing an in-frame stop codon that produces a polypeptide containing only the first 73 amino acids. A new ORF producing the last 353 amino acids also would be predicted in aberrant transcripts (Figure 8). Attempts to identify this aberrant transcript in 'Thompson', 'Ruby Red', 'Flame', and 'Star Ruby' grapefruit cultivars were unsuccessful due its high similarity of sequence with normal transcripts.

Figure 3- Alignment of the partial cDNA sequences of the wild type and mutant lycopene β-cyclases from ‘Duncan’, ‘Thompson’, ‘Ruby Red’, ‘Flame’, and ‘Star Ruby’ grapefruit cultivars. Nucleotides identical for all sequences in a given position are in white text on a black background. Dashes denote a gap in the nucleotide sequence. Black text in a gray background indicates the start codon. The alignment was created by using ClustalW Ver. 1.8. Numbers to the left denote the number of the nucleotide that starts the row.

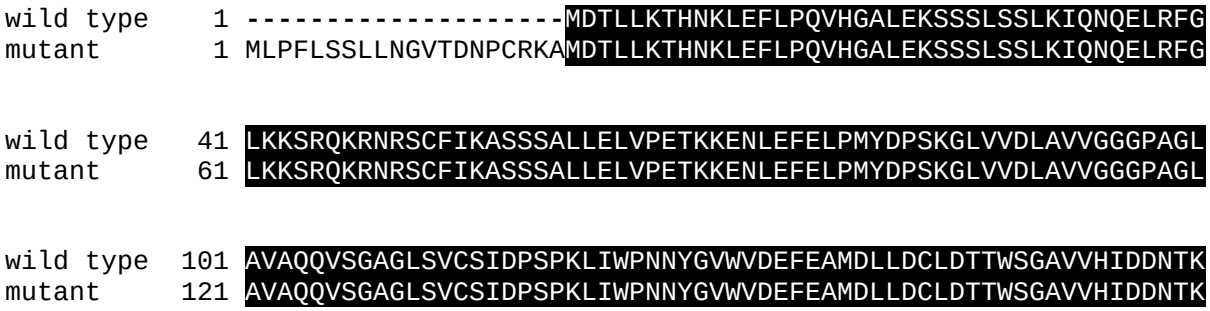


Figure 4- Alignment of deduced amino acid sequences of the wild type and mutant lycopene β-cyclase enzymes from ‘Duncan’, ‘Thompson’, ‘Ruby Red’, ‘Flame’, and ‘Star Ruby’ grapefruit cultivars. Residues identical for all sequences in a given position are in white text on a black background. Black text in a gray background denotes the amino acid residues added in the mutant protein. The alignment was created by using ClustalW Ver. 1.8. Numbers to the left denote the number of the amino acid residue that starts the row.

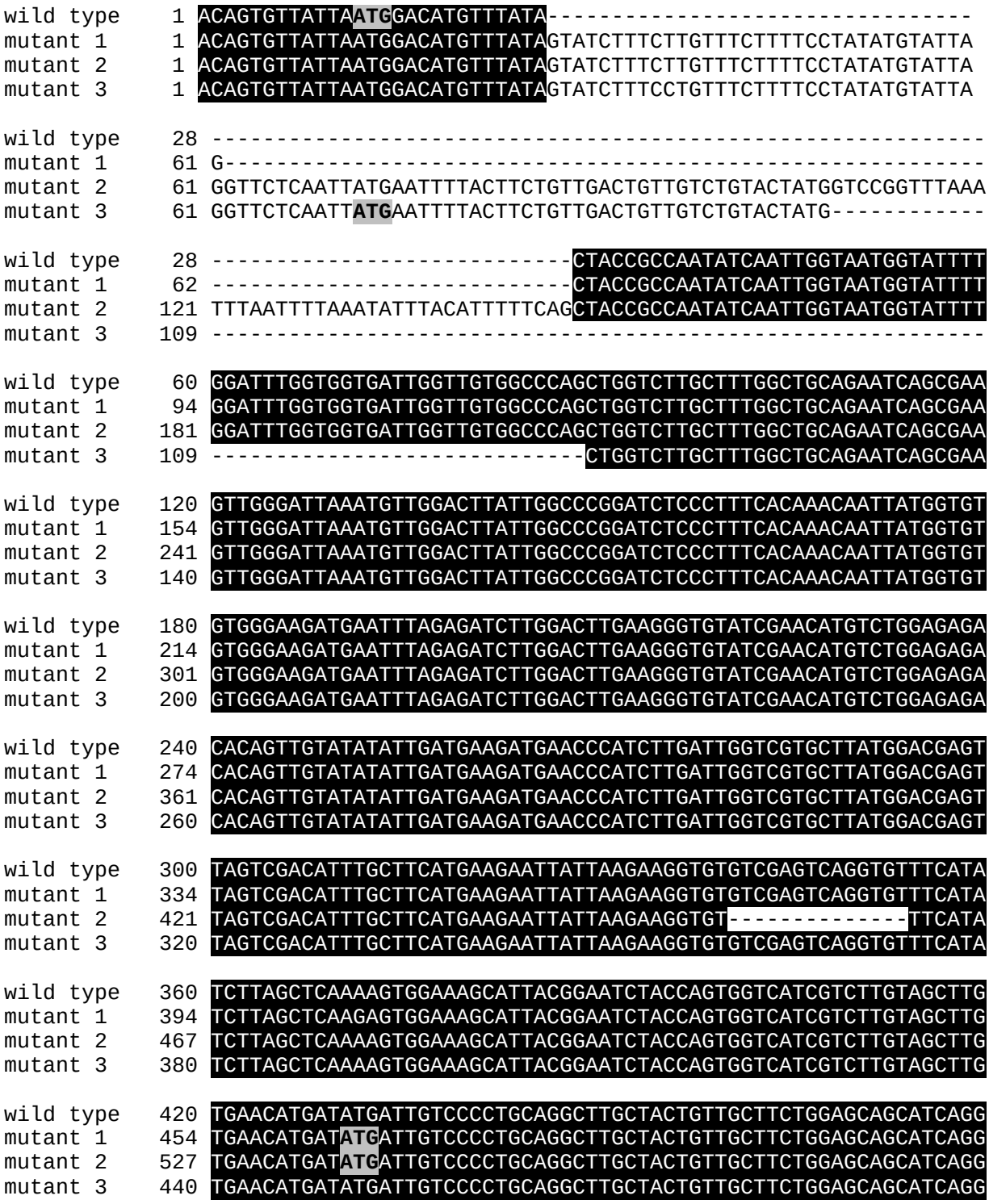


Figure 5- Alignment of the partial cDNA sequence of the wild type and mutant lycopene ε-cyclases from 'Duncan', 'Thompson', 'Ruby Red', 'Flame', and 'Star Ruby' grapefruit cultivars. Nucleotides identical for all sequences in a given position are in white text on a black background. Dashes denote a gap in the nucleotide sequence. Black text in a gray background indicates the start codon. The alignment was created by using ClustalW Ver. 1.8. Numbers to the left denote the number of the nucleotide that starts the row.

a

```
Duncan   615 GTT TTC ATG GAC TAC.. 702 TCT TCA ACA AGA GTT.. 1221 CTC TCA TCA GCC GAT..
             V   F   M   D   Y...      S   S   T   R   V...       L   S   S   A   D...
Thompson 615 GTT TTC GTG GAC TAC.. 702 TCT TCA GCA AGA GTT.. 1221 CTC TCA CCA GCC GAT..
             V   F   V   D   Y...      S   S   A   R   V...       L   S   P   A   D...
                     193                      222                       395
```

b

```
Duncan 351 GAA GAA TTA TTA AGA.. 750 GGT TTA CGT TTT GAC.. 810 CAG GTT TTG AAA ACT..
           E   E   L   L   R...      G   L   R   F   D...      Q   V   L   K   T...
Flame  351 GAA GAA TCA TTA AGA.. 750 GGT TTA CCT TTT GAC.. 810 CAG GTT TCG AAA ACT..
           E   E   S   L   R...      G   L   P   F   D...      Q   V   S   K   T...
                   105                      238                       258

Duncan 1107 TTC CTC TTT GGA CTA.. 1155 AGG ACA TTC TTT CGC..
            F   L   F   G   L          R   T   F   F   R...
Flame  1107 TTC CTC TCT GGA CTA.. 1155 AGG ACA CTC TTT CGC..
            F   L   S   G   L...       R   T   L   F   R...
                    357                    373

Duncan 1350 ACT TTA TAG TTA GTT TGT ATT TTC CAT ATT TCA GCC CTT GTT TGG TAT ATT
            T   L   *
Flame  1350 ACT TTA CAG TTA GTT TGT ATT TTC TAT ATT TCA GCC CTT GTT TGG TAT ATT
            T   L   Q   L   V   C   I   F   Y   I   S   A   L   V   W   Y   I
                437

Duncan 1401 TTG GAT TGC CAT ACG TGA

Flame  1401 TTG GAT TGC CAT ACA TGA
            L   D   C   H   T   *
```

c

```
Duncan     219 GAT GAA TTT  AGA GAT CTT GGA CTT GAA GGG TGT ATC GAA CAT GTC TGG AGA
               D   E   F    R   D   L   G   L   E   G   C   I   E   H   V   W   R
Ruby Red   219 GAT GAA TT-A GAG ATC TTG GAC TTG AAG GGT GTA TCG AAC ATG TCT GGA GAG
               D   E   L    E   I   L   D   L   K   G   V   S   N   M   S   G   E
                   60

Duncan     270 GAC ACA GTT GTA TAT ATT GAT GAA GAT GAA CCC ATC TTG A..
               D   T   V   V   Y   I   D   E   D   E   P   I   L   I..
Ruby Red   270 ACA CAG TTG TAT ATA TTG ATG AAG ATG AAC CCA TCT TGA..
               T   Q   L   Y   I   L   M   K   M   N   P   S   *
```

d

```
Duncan     612 ATG GTT TTC  ATG GAC TAC AGA GAC TGT ACT AAG CAA GAA GTT CCA TCT TTT
               M   V   F    M   D   Y   R   D   C   T   K   Q   E   V   P   S   F
Star Ruby  612 ATG GTT T-CA TGG ACT ACA GAG ACT GTA CTA AGC AAG AAG TTC CAT CTT TTG
               M   V   S    W   T   T   E   T   V   L   S   K   K   F   H   L   L
                       191
Duncan     663 GAA TCT GAC AAT CCA ACA TTT CTT TAT GTC ATG CCC ATG TCT TCA ACA AGA
               E   S   D   N   P   T   F   L   Y   V   M   P   M   S   S   T   R
Star Ruby  663 AAT CTG ACA ATC CAA CAT TTC TTT ATG TCA TGC CCA TGT CTT CAA CAA GAG
               N   L   T   I   Q   H   F   F   M   S   C   P   C   L   Q   Q   E

Duncan     714 GTT TTC TTT GAG GAA ACT TGT TTG GCA TCG AAA GAT GGT TTA CGT TTT GAC
               V   F   F   E   E   T   C   L   A   S   K   D   G   L   R   F   D
Star Ruby  714 TTT TCT TTG AGG AAA CTT GTT TGG CAT CGA AAG ATG GTT TAC GTT TTG ACA
               F   S   L   R   K   L   V   W   H   R   K   M   V   Y   V   L   T

Duncan     765 ATA TTG AA..
               I   L   K..
Star Ruby  765 TAT TGA..
               Y   *
```

Figure 6- Nucleotide and amino acid comparison of the lycopene ε-cyclase genes between the different grapefruit cultivars. a, Alignment of the partial cDNA sequence from ‘Duncan’and ‘Thompson’ showing three nucleotide differences (boxed) in ‘Thompson’; b, Alignment of the partial cDNA sequence from ‘Duncan’ and ‘Flame’ showing six nucleotide differences (boxed) in ‘Flame’; c, Alignment of the partial cDNA sequence from ‘Duncan’ and ‘Ruby Red’ showing a single-base deletion (boxed) in ‘Ruby Red’; d, Alignment of the partial cDNA sequence from ‘Duncan’ and ‘Star Ruby’ showing a single-base deletion (boxed) in ‘Star Ruby’;. The amino acid sequence created by the changes is shown in italics.

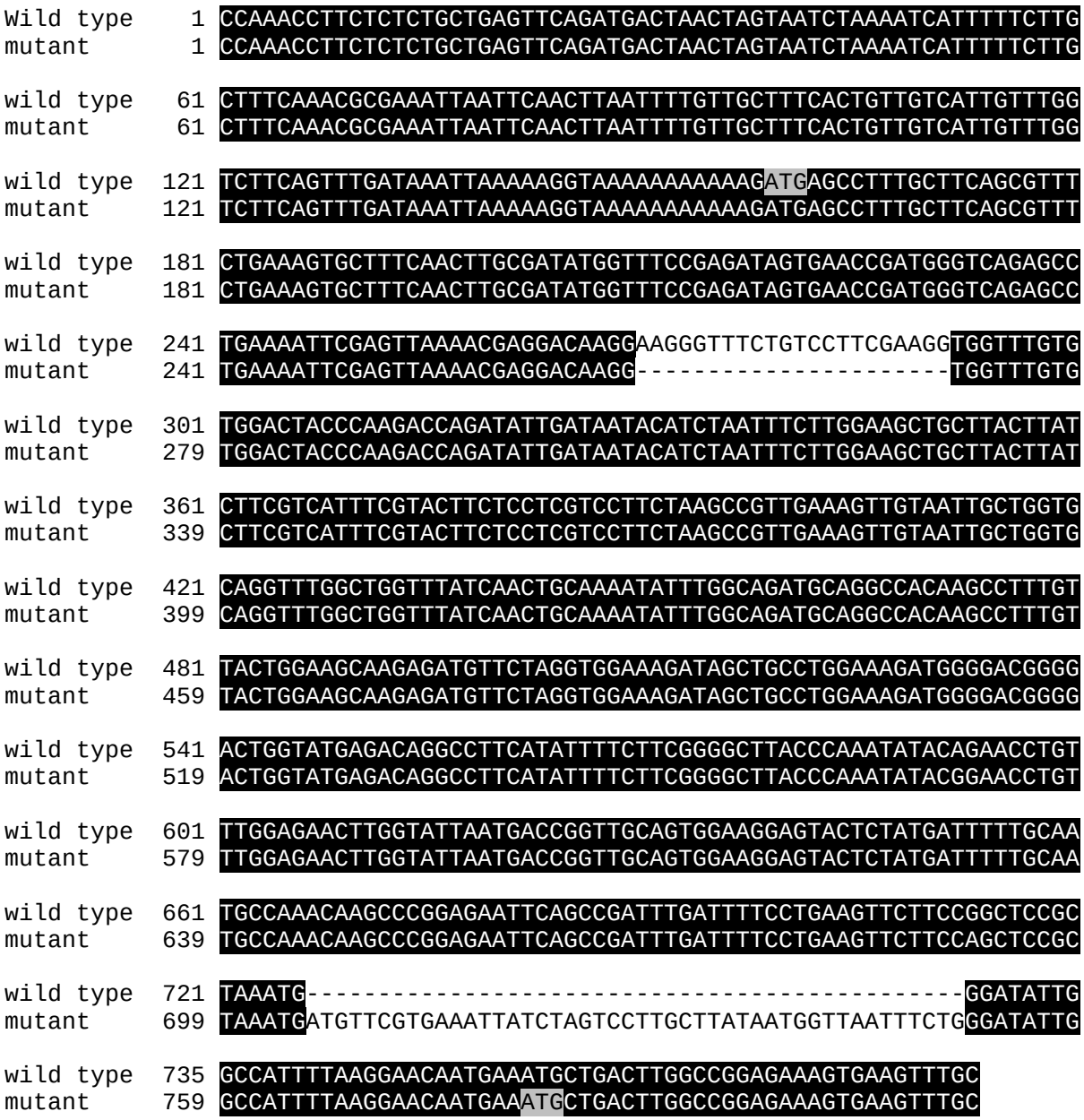


Figure 7- Alignment of the partial cDNA sequences of the wild type and mutant phytoene desaturase from ‘Duncan’ grapefruit. Nucleotides identical for all sequences in a given position are in white text on a black background. Dashes denote a gap in the nucleotide sequence. Black text in a gray background indicates the start codon. The alignment was created by using ClustalW Ver. 1.8. Numbers to the left denote the number of the nucleotide that starts the row.

```
wild type    1 MSLCFSVSESAFNLRYGFRDSEPMGQSLKIRVKTRTRKGFCPSKVVCVDYPRPDIDNTSN
mutant       1 ------------------------------------------------------------

wild type   61 FLEAAYLSSSFRTSPRPSKPLKVVIAGAGLAGLSTAKYLADAGHKPLLLEARDVLGGKIA
mutant       1 ------------------------------------------------------------

wild type  121 AWKDGDGDWYETGLHIFFGAYPNIQNLFGELGINDRLQWKEYSMIFAMPNKPGEFSRFDF
mutant       1 ------------------------------------------------------------

wild type  181 PEVLPAPLNGILAILRNNEMLTWPEKVKFAIGLLPAIIGGQAYVEAQDGLTVQEWMRKQG
mutant       1 -------------------MLTWPEKVKFAIGLLPAIIGGQAYVEAQDGLTVQEWMRKQG

wild type  241 VPDRVTTEVFIAMSKALNFINPDELSMQCILIALNRFLQEKHGSKMAFLDGNPPERLCLP
mutant      42 VPDRVTTEVFIAMSKALNFINPDELSMQCILIALNRFLQEKHGSKMAFLDGNPPERLCLP

wild type  301 IVEHIQSLGGEVRLNSRVQKIELNDDGTVKNFLLTNGNVIDGDAYVFATPVDILKLQLPE
mutant     102 IVEHIQSLGGEVRLNSRVQKIELNDDGTVKNFLLTNGNVIDGDAYVFATPVDILKLQLPE

wild type  361 NWKEMAYFKRLEKLVGVPVINIHIWFDRKLKNTYDHLLFSRSPLLSVYADMSLTCKEYYN
mutant     162 NWKEMAYFKRLEKLVGVPVINIHIWFDRKLKNTYDHLLFSRSPLLSVYADMSLTCKEYYN

wild type  421 PNQSMLELVFAPAEEWISCSDSEIIDATMKELAKLFPDEISADQSKAKIVKYHVVKTPRS
mutant     222 PNQSMLELVFAPAEEWISCSDSEIIDATMKELAKLFPDEISADQSKAKIVKYHVVKTPRS

wild type  481 VYKTIPNCEPCRPLQRSPVEGFYLAGDYTKQKYLASMEGAVLSGKLCAQAIVQDYVLLAA
mutant     282 VYKTIPNCEPCRPLQRSPVEGFYLAGDYTKQKYLASMEGAVLSGKLCAQAIVQDYVLLAA

wild type  541 RGKGRLAEASMC
mutant     342 RGKGRLAEASMC
```

Figure 8- Alignment of deduced amino acid sequences of the wild type and mutant phytoene desaturase enzymes from ‘Duncan’ grapefruit. Residues identical for all sequences in a given position are in white text on a black background. The alignment was created by using ClustalW Ver. 1.8. Numbers to the left denote the number of the amino acid residue that starts the row.

Expression analysis of the isolated carotenoid biosynthetic genes during grapefruit fruit development

Transcriptional regulation of carotenoid biosynthetic genes has been described as the major regulatory mechanism of carotenoid accumulation in flowers and fruits from several species (Ronen et al., 1999). In order to determine whether fruit color variation between the grapefruit cultivars is caused by differential gene regulation, this study was undertaken aiming to characterize the expression of the isolated carotenoid biosynthetic genes in two cultivars, 'Duncan' (a white fleshed fruit cultivar) and 'Flame' (a deep red fleshed fruit cultivar). Initial attempts by Northern hybridization were done using up to 20 μg of total fruit RNA. The levels of the transcripts were so low that they could not be detected by this method (Melton, 1998). The RT-PCR technique provided the level of sensitivity required to study these rare transcripts using as little as 2 μg total fruit RNA. Samples of albedo and juice vesicles/segment wall tissue from fruits of 'Duncan' and 'Flame' were harvested during the time course of fruit development (see material and methods). Total RNA was isolated, DNase-treated, and used as template in reactions of reverse transcription. Relative quantitative RT-PCR was used to quantify the levels of expression of the isolated carotenoid biosynthetic genes. Linear range analysis of the transcripts revealed an optimal number of PCR cycles of 35 (results not shown). The reactions were normalized against 18S rRNA, which was co-amplified in the same linear range as the mRNAs under study using the 2:8 18S primers:competimers optimal ratio (Competimer^{TM} technology, Ambion). The corrected relative values in each tissue were compared between the two grapefruit cultivars for an estimate of the relative expression between the cultivars.

Expression patterns of PSY, PDS, and ZDS varied between both tissues and both cultivars (Figure 9; Figure 10). In addition, the patterns were complex, with transcript levels often rising and falling at varying times during fruit maturation. In albedo, there was in general a higher transcript abundance for these three genes in 'Duncan' than in 'Flame', especially at the beginning of the fruit development. The same was generally true in endocarp, although the pattern

was not as obvious. Also in endocarp, there was a sharp rise in PSY levels in both cultivars in the middle of the ripening period, when color was at its highest. It was also striking that in both cultivars the expression of PDS and ZDS in both tissues practically disappeared during the same time period. In contrast, levels of LCY-B transcript changed minimally during fruit development in both tissues and both grapefruit cultivars, implying constitutive expression.

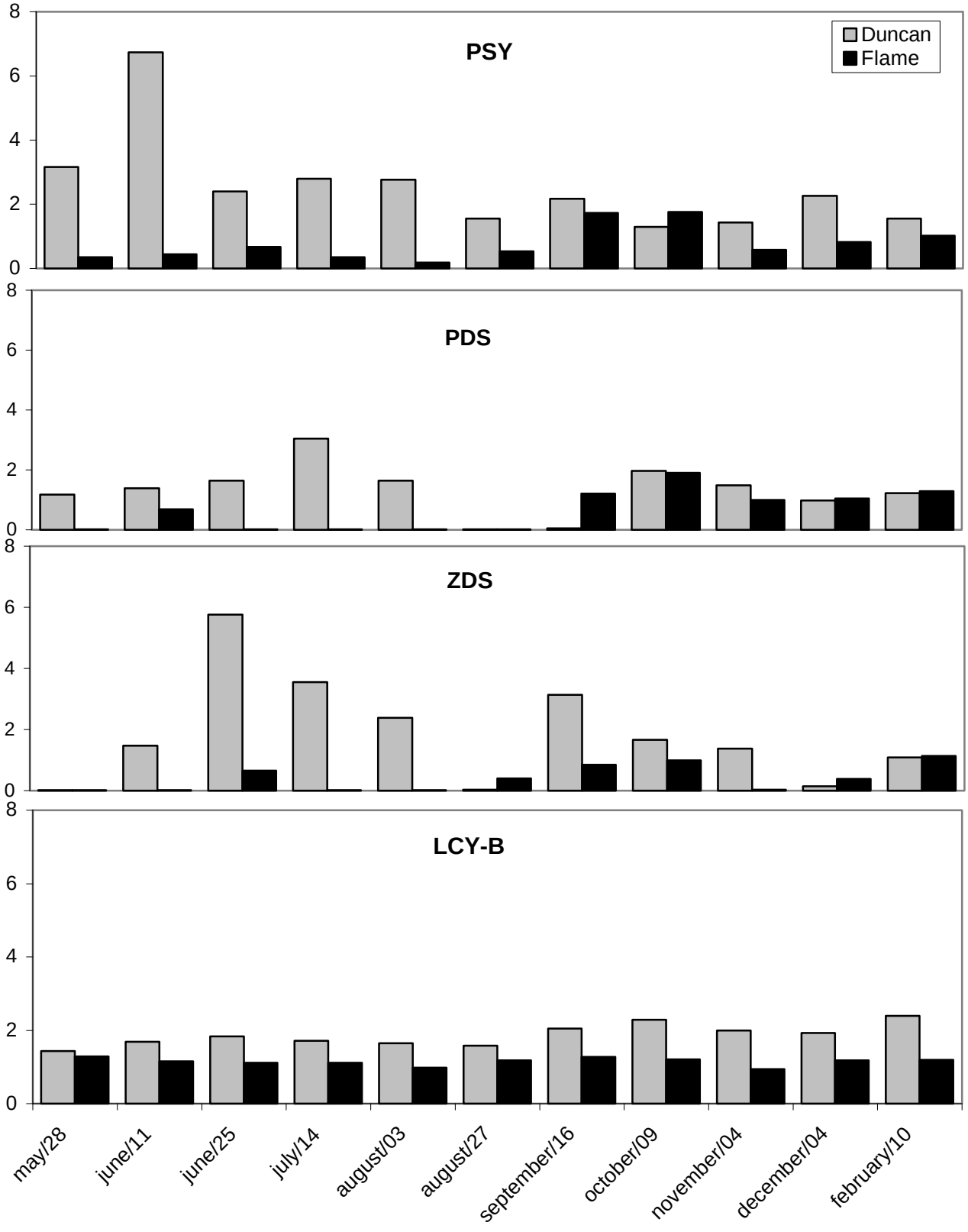


Figure 9- Developmental expression of the carotenoid biosynthetic genes in albedo fruit tissue from 'Duncan' (white) and 'Flame' (deep red) grapefruit cultivars. Bar graphs represent expression levels of the indicated genes relative to expression of the 18S rRNA. Abbreviations: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LCY-B, lycopene β-cyclase.

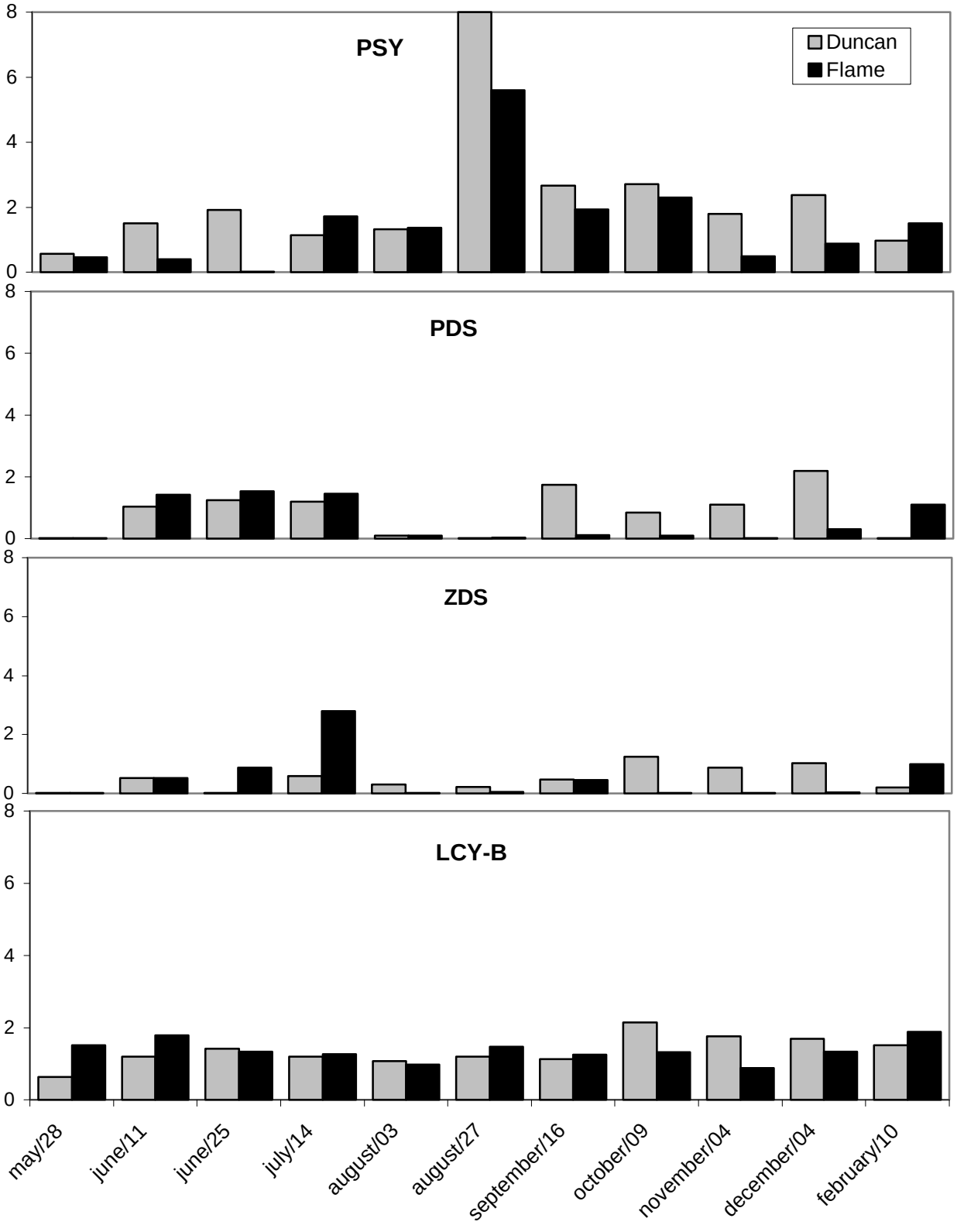


Figure 10- Developmental expression of the carotenoid biosynthetic genes in juice vesicles/segment wall tissue from 'Duncan' (white) and 'Flame' (deep red) grapefruit cultivars. Bar graphs represent expression levels of the indicated genes relative to expression of the 18S rRNA. Abbreviations: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LCY-B, lycopene β-cyclase.

DISCUSSION

Little is known about the molecular biology of the carotenoid biosynthetic pathway in *C. paradisi* and what process produces the differences in color between the cultivars. Therefore we isolated the genes involved in the early steps of the carotenoid biosynthetic pathway from *C. paradisi*. The RT-PCR technique was effectively employed to identify and clone full-length cDNA sequences from all of the carotenoid biosynthetic genes described in this study in a short time period and using as little as 2 μg total RNA.

It was somewhat surprising to find two or more different transcripts for three of the five genes characterized in this study, but still more surprising that in each case one or more of the transcripts was aberrant, so that the production of a nonfunctional protein would be predicted. However, there are several lines of evidence that indicate that this is indeed the case and that the identified sequences were not an artifact of cloning or sequencing. First, homologous transcripts were identified for each gene from five different grapefruit cultivars and two kind of tissues. In the case of lycopene-β-cyclase and lycopene-ε-cyclase, normal and aberrant transcripts were identified from each cultivar and tissue. In the cases where there were differences between cultivars, the same transcripts were identified from the two different tissue sources.

Also, during the course of this research, genes involved in carotenoid production were identified by others in some other citrus types. A cDNA for PSY was cloned from Satsuma mandarin (*C. unshiu* Marc.) by using a PCR product to

screen a fruit cDNA library (Ikoma et al., 2001). Three positive clones were identified and the longest one was sequenced. The sequence of the cDNA was 98% identical at the nucleotide level to that of the normal transcript identified here in grapefruit. Southern analysis indicated that the PSY clone was homologous to a few sequences in the mandarin genome. PDS cDNAs were isolated from mandarin and sweet orange fruit (by identifying two positive clones in mandarin; Kita et al., 2001). The cDNAs were 98% homologous to each other and both were 98% homolgous to that of the normal transcript of grapefruit. Again, a small gene family was predicted. ZDS has also been cloned from mandarin and sweet orange and the sequences are again 98% identical to that of the normal transcript of grapefruit. No further characterization of this gene has been reported. A cDNA that has homology to carotenoid associated proteins from other species, designated citPAP, was isolated from Satsuma mandarin during an expressed sequence tag screen and Southern analysis suggested that it was a low copy number gene, but that related sequences were also present (Moriguchi et al., 1998). Thus, in these studies of carotenoid biosynthetic genes in other citrus types, relatively few and the longest clones were sequenced, giving rise to nucleotide sequences quite similar to those found in grapefruit that we have designated as normal and that are predicted to encode a functional protein that could be imported into plastids. However, in all cases where they have been examined by Southern analysis, the cloned sequences have homology to several sequences in citrus, all of which were not identified in the screens.

Finally, Kim et al. (2001) screened Satsuma mandarin fruit and leaf cDNA libraries with a PCR product made from primers degenerate for conserved sequences of the β-carotene hydroxylase gene. Two cDNAs were isolated, which differed in six nucleotides and therefore three amino acids. The base changes were within the ORF; the 5’ and 3’ UTRs were the same, so that the sequences appeared to correspond to alleles of the same gene. Thus there is precedent in citrus for what we observed in grapefruit.

It is difficult to compare levels and types of carotenoids in the various citrus species. The literature describing carotenoid biosynthesis is very old and

the earlier reports of carotenoid content may contain artifacts (Stewart, 1977). Also, carotenoid levels are expressed differently in different reports, making comparisons difficult. Finally, the tissues used for extraction may be quite different. For example, citrus peel consists of two tissue types, the spongy white albedo and the flavedo, which is the colored part of the peel. Various researchers refer to peel, and it is unclear whether this means both tissues or just the flavedo.

However, some hypotheses can be made, considering our data and what is in the literature. Total carotenoid content in the flavedo of mature white grapefruit is more than 50-fold lower than that of oranges and mandarins (Gross, 1977). Perhaps the aberrant transcripts present at multiple loci greatly decrease flux in the carotenoid biochemical pathway in general in both white and colored grapefruit.

The single-base deletions found in the "normal" lycopene ε-cyclase transcripts of 'Ruby Red' and 'Star Ruby' may be related to fruit color differentiation between the grapefruit cultivars. The deletions cause frame-shift resulting in truncated polypeptides. Even though similar frame-shift mutations were not found in 'Thompson' and 'Flame' due the high sequence similarity of the transcripts, the presence of different lycopene ε-cyclase isoforms suggested by our results indicates that the same situation may occur in these cultivars. The presence of additional lycopene ε-cyclase isoforms has also been suggested in marigold (Moehs et al., 2001).

The transcripts present for PDS in grapefruit differed between white 'Duncan' and the pink- and red-colored grapefruit; apparently, only 'Duncan' had an aberrant transcript for this gene. The colorless hydrocarbons phytoene and phytofluene account for three-fourths of the carotenoid content of white grapefruit flavedo (Yokoyama and White, 1967). Surprisingly, Ruby Red grapefruit peel also accumulated large amounts of these noncolored compounds (60%); lycopene accounted for only 11% of the carotenoids present (Curl and Bailey, 1957). When only colored carotenoids were considered, 87% of the carotenoids present in white flavedo were xanthophylls. Thus, the pathway could not be completely shut down at the level of PDS in white grapefruit, but perhaps

a partial blockage in flux there prevented the accumulation of lycopene. The arabidopsis recessive mutation *immutans* affects the activity of the PTOX that is associated with PDS, yielding an albino, photobleached phenotype (that accumulates phytoene) interrupted by green sectors (Carol et al., 1999). The same mutant in tomato is called *ghost* (Carol and Kuntz, 2001). This group suggests that the mutation may not totally block PDS function because carotenoids are present in the fruit of *ghost* and the green sectors of *immutans*. Tomatoes heterozygous for *ripening-inhibitor* remain firm and ripen over a protracted period, presumably due to reduced levels of functional RIN protein (Vrebalov et al., 2002).

The expression analysis of the isolated carotenoid biosynthetic genes indicated complex expression patterns for PSY, PDS, and ZDS. The patterns varied between the two grapefruit cultivars and the two tissues examined. However, in no case was there a clear upregulation of the early genes in the pathway during the entire ripening period as has been described in the climateric tomato fruit (Ronen et al., 1999). This may be because the levels of various carotenoids present change noncoordinately as ripening progresses. In oranges in both flavedo and endocarp, total carotenoid levels continue to increase in ripening fruit throughout the maturation period after what may be a very sharp increase at color break (the stage where chlorophyll disappears and carotenogenesis becomes apparent) (Gross, 1977). In white grapefruit there is no further synthesis of colored carotenoids during ripening after the disappearance of chlorophyll, but there is a marked synthesis of phytoene (Yamamoto and White, 1967). In pink- and red-fleshed grapefruit, the amounts of colored carotenoid species that accumulate in the endocarp change during maturation. Lycopene accumulates to its highest level approximately mid-way during the ripening period and then decreases, while β-carotene levels continue to increase for much longer, gradually leveling off (Ting and Deszyck, 1957; Lime et al., 1954; Purcell, 1959a). The accumulation patterns of both compounds are the same in red and pink grapefruit, but the relative amounts are higher in red grapefruit and pink grapefruit contain more β-carotene (Khan and Mackinney,

1956; Lime et al., 1956; Curl and Bailey, 1957). There were also changes in the levels of different carotenoids in the endocarp and flavedo of ‘Shamouti’ orange during ripening (Eilati et al., 1969; Gross, 1977). It has been hypothesized that lycopene synthesis and lycopene degradation are occurring simultaneously in red grapefruit; Purcell found that $^{14}CO_2$ was incorporated into lycopene after the lycopene level began to decline (Purcell et al., 1961). Thus, regulation of synthesis of the different carotenoids may be very complex.

There was a spike in PSY expression in the endocarp of both cultivars midway through the ripening period. Also in both cultivars, expression of PDS and ZDS became almost undetectable in the middle of the season in both tissues, when both juice vesicles and peel were most highly colored in ‘Flame’. The levels then increased again towards the end of the season. The same pattern was observed in Satsuma mandarin for CitPAP in albedo tissue but not other tissues (Moriguchi et al., 1998). A similar pattern was also observed for PSY in endocarp vesicles of Satsuma mandarin (Ikoma et al., 2001). However, in another experiment with Satsuma, the level of PSY increased during fruit maturation with a large increase late in maturity (Kita et al., 2001).

The expression pattern of the isolated carotenoid biosynthetic genes was different between the grapefruit fruit tissues in both cultivars, suggesting a differential expression of the transcripts within the fruit tissues. The two tissues accumulate different levels of carotenoid species. For example, the carotenoids in ‘Ruby Red’ peel were 47% phytoene, 14% phytofluene, 11% lycopene, and 7% β-carotene, while the same carotenoids accumulated in the interior fruit tissues at levels of 16%, 4%, 40%, and 27%, respectively (Curl and Bailey, 1957). Differences in expression between fruit tissues were also noted for PDS transcripts in *C. unshiu*, which displayed different expression patterns in peel and juice sacs/segment epidermis (Kita et al., 2001). Also, whereas in albedo the accumulation pattern of the phytoene synthase and phytoene desaturase transcripts was roughly similar in the grapefruit cultivars, in juice vesicles it was different, indicating a non-coordinate regulation of the phytoene synthase and phytoene desaturase genes in this fruit tissue. Changes in the expression of the

lycopene β-cyclase gene were not observed in the fruit tissues of the grapefruit cultivars. A similar result for β-carotene hydroxylase transcripts has been observed in *C. unshiu*, which changed minimally during fruit and leaf development (Kim et al., 2001). It can be suggested that lycopene β-cyclase and β-carotene hydroxylase products do not catalyze the rate-limiting step in the carotenoid pathway of ripening citrus fruits. Ours results indicate that the phytoene synthase is the rate-limiting enzyme of carotenoid biosynthesis in ripening grapefruit fruits, since the pattern of lycopene accumulation during fruit development of colored grapefruits demonstrated in previous studies, with maximum concentration near the end of August followed by a sharp decline until the middle of November (Lime et al., 1954; Ting and Deszyck, 1957; Purcell, 1959a), is strictly correlated with the expression levels of this gene in juice vesicles. Due its position as the first commited step in the carotenoid pathway, it is expected that phytoene synthase enzyme controls the flux into the pathway, as has been demonstrated in ripening tomato fruits (Fraser et al., 1994), canola (Shewmaker et al., 1999), and marigold flowers (Moehs et al., 2001).

In summary, we have isolated five genes from the carotenoid biosynthetic pathway of *C. paradisi*. The results indicate that frame-shift mutation and not transcriptional regulation may be responsible for the color differentiation between the grapefruit cultivars, with phytoene desaturase and/or lycopene ε-cyclase being the candidate genes for fruit color differences. Further work will focus these two genes as well as the identification of new genes for the steps in the pathway we have examined, since multiple genes or small gene families are common for several of the steps involved in carotenoid biosynthesis (Okada et al., 1999; Bartley and Scolnik, 1993).

Acknowledgments

We are grateful to Dr Joseph Hirschberg for providing the plasmids pACCRT-EIB and Crt L. We also appreciate the technical assistance provided by Ms Kim Ruesch and Dr Mike McCaffery. This research was supported by CAPES Foundation (Coordination of Higher Education and Graduate Training), Brasilia/Brazil, and Institute of Food and Agricultural Sciences at the University of Florida, USA. We gratefully acknowledge the 'Sandwich' Ph.D. scholarship to M.G.C. Costa by the CAPES Foundation, Brasilia/Brazil (process # BEX0600/00-8).

REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman D.J. 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403-410.

Bartley, G.E. and Scolnik, P.A. 1993. cDNA cloning, expression during development and genome mapping of Psy-2, a second tomato gene encoding phytoene synthase. J. Biol. Chem. 267: 5036-5039.

Bartley, G.E. and Scolnik, P.A. 1995. Plant carotenoids: pigments for photoprotection, visual attraction, and human health. Plant Cell 7: 1027-1038.

Britton, G. 1995. Structure and properties of carotenoids in relation to function. FASEB J. 9: 1551-1558.

Cameron, J.W., Soost, R.K. and Olson, E.O. 1964. Chimeral basis for color in pink and red grapefruit. J. Hered. 55: 23-28.

Carol, P. and Kuntz, M. 2001. A plastid terminal oxidase comes to light: implications for carotenoid biosynthesis and chlororespiration. Trends Plant Sci. 6:31-36.

Carol, P., Stevenson, D., Bisanz, C., Breitenbach, J., Sandmann, G., Mache, R., Coupland, G. and Kuntz, M. 1999. Mutations in the Arabidopsis gene *IMMUTANS* cause a variegated phenotype by inactivating a chloroplast terminal oxidase associated with phytoene desaturation. Plant Cell 11:57-68.

Chang, S., Puryear, J. and Cairney, J. 1993. A simple and effective method for isolating RNA from pine trees. Plant Mol. Biol. Rep. 11: 113-116.

Cunningham, F.X. Jr. and Gantt, E. 1998. Genes and enzymes of carotenoid biosynthesis in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49: 557-583.

Cunningham, F.X. Jr., Pogson, B., Sun, Z., McDonald, K.A., DellaPenna, D. and Gantt, E. 1996. Functional analysis of the and lycopene cyclase enzymes reveals a mechanism for control of cyclic carotenoid formation. Plant Cell 8:1613-1626.

Curl, A.L. and Bailey, G.F. 1956a. The carotenoids of Ruby Red grapefruit. Food Res. 22: 63-68.

Curl, A.L. and Bailey, G.F. 1956b. Part 1 – Comparison of carotenoids of Valencia orange peel and pulp. Agricult. Food Chem. 4:156-159.

Eilati, S.K., Monselise, S.P. and Budowski, P. 1969. Seasonal development of external color and carotenoid content in the peel of ripening 'Shamouti' oranges. J. Amer. Soc. Hort. Sci. 94:346-348.

Emanuelsson, O., Nielsen, H. and von Heijne, G. 1999. ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Prot. Sci. 8: 978-984.

Fraser, P.D., Truesdale, M.R., Bird, C.R., Schuch, W. and Bramley, P.M. 1994. Carotenoid biosynthesis during tomato fruit development. Plant Physiol. 105: 405-413.

Gmitter, F.G. Jr. 1995. Origin, evolution, and breeding of the grapefruit. Plant Breed. Rev. 13:345-363.

Goodwin, T.W. 1980. The Biochemistry of the Carotenoids, Vol. 1, 2nd ed. Chapman and Hall, London, NY.

Gross, J. 1977. Carotenoid pigments in citrus. In: S. Nagy, P.E. Shaw and M. Veldhuis (Eds.), Citrus Science and Technology, Vol. 1. Avi. Pub. Co., Westport, pp. 302-354.

Hirschberg, J. 2001. Carotenoid biosynthesis in flowering plants. Curr. Opin. Plant Biol. 4: 210-218.

Hugueney, P., Badillo A., Chen H.-C., Klein, A., Hirschberg, J., Camara, B. and Kuntz, M. 1995. Metabolism of cyclic carotenoids: a model for the alteration of this biosynthetic pathway in *Capsicum annuum.* Plant J. 8:417-424.

Hugueney, P., Römer, S., Kuntz, M. and Camara, B. 1992. Characterization and molecular cloning of a flavoprotein catalyzing the synthesis of phytofluene and -carotene in *Capsicum* chromoplasts. Eur. J. Biochem. 209:399-407.

Ikoma, Y., Komatsu, A., Kita, M. Ogawa, K., Omura, M., Yano, M. and Moriguchi, T. 2001. Expression of a phytoene synthase gene and characteristic carotenoid accumulation during citrus fruit development. Physiol. Plant. 111:232-238.

Isaacson, T., Ronen, G., Zamir, D. and Hirschberg, J. 2002. Cloning of *tangerine* from tomato reveals a carotenoid isomerase essential for the production of - carotene and xanthophylls in plants. Plant Cell 14:333-342.

Josse, E.-M., Simkin, A.J., Gaffé, J., Labouré, A.-M., Kuntz, M. and Carol, P. 2000. A plastid terminal oxidase associated with carotenoid desaturation during chromoplast differentiation. Plant Physiol. 123:1427-1436

Khan, M. and Mackinney, G. 1953. Carotenoids in grapefruit, *Citrus paradisi*. Plant Physiol. 28: 550-552.

Kim, I.-J., Ko, K.-C., Kim, C.-S. and Chung, W.-I. 2001. Isolation and expression patterns of a cDNA encoding phytoene synthase in *Citrus*. J. Plant Physiol. 158: 795-800.

Kita, M., Komatsu, A., Omura, M., Yano, M., Ikoma, Y. and Moriguchi, T. 2001. Cloning and expression of CitPDS1, a gene encoding phytoene desaturase in citrus. BioSci. Biotech. Biochem. 65: 1424-1428.

Lee, H.S. and Castle, W.S. 2001. Seasonal changes of carotenoid pigments and color in Hamlin, Earlygold, and budd blood orange juices. J. Agric. Food Chem. 49:877-882.

Lime, B.J., Stephens, T.S. and Griffiths, F.P. 1954. Processing characteristics of colored Texas grapefruit. I. Color and maturity studies of Ruby Red grapefruit. Food Technol. 8: 566-569.

Mayne, S.T. 1996. Beta-carotene, carotenoids, and disease prevention in humans. FASEB J. 10: 690-701.

Melton, J.R. 1998. The determination of the molecular basis for color differences in the fruit of *Citrus paradisi* by several methods. Master of Science dissertation, University of Florida, Gainesville, FL.

Moehs, C.P., Tian, L., Osteryoung, K.W. and DellaPenna, D. 2001. Analysis of carotenoid biosynthetic gene expression during marigold petal development. Plant Mol. Biol. 45: 281-293.

Moore, G.A. 2001. Oranges and lemons: clues to the taxonomy of *Citrus* from molecular markers. TIG 17:536-540.

Moriguchi, T., Kita, M., Endo-Inagaki, T., Ikoma, Y. and Omura, M. 1998. Characterization of a cDNA homologous to carotenoid-associated protein in citrus fruits. Biochim. Biophys. Acta 1442:334-338

Niyogi, K.K. 1999. Photoprotection revisited. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50: 391-417.

Okada, K., Kamiya, Y., Saito, T., Nakagawa, T. and Kaawamukai, M. 1999. Localization and expression of geranylgeranyl diphosphate synthases in *Arabidopsis thaliana*. Annual Meeting of the American Society of Plant Physiologists, Baltimore, MD.

Park, H., Kreunen, S.S., Cuttriss, A.J., Della Penna, D. and Pogson, B.J. 2002. Identification of the carotenoid isomerase provides insight into carotenoid biosynthesis, prolamellar body formation, and photomorphogenesis. Plant Cell 14:321-332.

Pecker, I., Gabbay, R., Cunningham, F.X. Jr. and Hirschberg, J. 1996. Cloning and characterization of the cDNA for lycopene beta-cyclase from tomato reveals decrease in its expression during fruit ripening. Plant Mol. Biol. 30:807-819.

Purcell, A.E. 1959a. Seasonal development of carotene and lycopene in grapefruit. J. Rio Grande Val. Hort. Soc. 13: 45-53.

Purcell, A.E. 1959b. Carotenoid pigment formation in colored grapefruit. J. Rio Grande Val. Hort. Soc. 13: 39-44.

Purcell, A.E, Carra, J.H. and deGruy, I.V. 1961. The development of chromoplasts and carotenoids in colored grapefruit. Proc. Amer. Soc. Hort. Sci. 82: 123-127.

Römer, S., Hugueney, P., Bouvier, F., Camara, B. and Kuntz, M. 1993. Expression of the genes encoding the early carotenoid biosynthetic enzymes in *Capsicum annuum*. Biochem. Biophys. Res. Commun. 196: 1414-1421.

Ronen, G. Carmel-Goren, L., Zamir, D. and J. Hirschberg. 2000. An alternative pathway to -carotene formation in plant chromoplasts discovered by map-based cloning of *Beta* and *old-gold* color mutations in tomato. Proc. Natl. Acad. Sci. 97:11102-11107.

Ronen, G., Cohen, M., Zamir, D. and Hirschberg, J. 1999. Regulation of carotenoid biosynthesis during tomato fruit development: expression of the gene lycopene epsilon-cyclase is down-regulated during ripening and is elevated in the mutant *Delta*. Plant J. 17: 341-351.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, NY.

Schledz, M., Al-Babili, S., von Liting, J., Haubruck, H., Rabbani, S., Kleinig, H. and Beyer, P. 1996. Phytoene synthase from *Narcissus pseudonarcissus*: functional expression, galactolipid requirement, topological distribution in chromoplast and induction during flowering. Plant J. 10: 781-792.

Shewmaker, C.K., Sheehy, J.A, Daley, M., Colburn, S. and Ke, DY. 1999. Seed specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. Plant J. 20: 401-412.

Stewart, I. 1977. Citrus color – a review. Proc. Int. Soc. Citriculture 1:308-311.

Stewart, I. and Wheaton, T.A. 1973. Carotenoids in citrus. Congresso Mundial de Citricultura, O. Carpena (ed.), Vol. 2, pp 325-330.

Ting, S.V. and Deszyck, E.J. 1957. The internal color and carotenoid pigments of Florida red and pink grapefruit. Am. Soc. Hort. Sci. 71: 271-277.

Vrebalov, J., Ruezinsky, D., Padmanabhan, V., White, R., Medrano, D., Drake, R., Schuch, W. and Giovannoni, J. 2002. A MADS-box gene necessary for

fruit ripening at the tomato *ripening-inhibitor (rin)* locus. Science 296:343-346.

Wetzel, C.M. and Rodermel, S.R. 1998. Regulation of phytoene desaturase expression is independent of leaf pigment content in *Arabidopsis thaliana*. Plant Mol. Biol. 37:1045-1053.

Yokoyama, H. and White, M.J. 1967. Carotenoids in the flavedo of Marsh seedless grapefruit. J. Agr. Food Chem. 15: 693-696.

Zeevaart, J.A.D. and Creelman, R.A. 1988. Metabolism and physiology of abscisic acid. Annu. Rev. Plant Physiol. Plant Mol. Biol. 39: 439-473.

CAPÍTULO II

AN EXAMINATION OF THE MORPHOGENIC GRADIENTS OF ADVENTITIOUS BUD AND SHOOT REGENERATION IN EPICOTYL EXPLANTS OF CITRUS

ABSTRACT

The *in vitro* responses of epicotyl explants from 'Cravo' rangpur lime (*Citrus limonia* Osb.), 'Foster' grapefruit (*C. paradisi* Macf.), and 'Pera' sweet-orange [*C. sinensis* (L.) Osb.] were characterized, which have not been previously reported. Further analysis was performed in 'Cravo' rangpur lime and 'Foster' grapefruit aiming to verify the *in vitro* morphogenesis of five distinct regions of the epicotyl under different treatments. It was observed the same general pattern of morphogenic gradient along the epicotyl axis in both citrus cultivars, with higher organogenic response as the distance of the explants from cotyledonary node increased. This morphogenic gradient was influenced by factors related to plant material, composition of the culture medium, and conditions of incubation. The relative importance of these factors in the pattern of expression of the morphogenic gradient along the epicotyl axis in citrus is discussed.

Key words: Citrus; 'Cravo' rangpur lime; 'Foster' grapefruit; 'Pera' sweet-orange; morphogenic gradients; organogenesis; shoot regeneration.

Abbreviations: *BAP:* 6-benzylaminopurine · *NAA:* naphthaleneacetic acid

INTRODUCTION

In vitro regeneration of citrus has been a subject of consistent research for a long time because of the economical importance of the genus and, most recently, because of the potential for further improvement via genetic manipulation. Citrus *in vitro* regeneration has been described in several species from different explant sources, including stem and epicotyl segments (Grinblat, 1972; Edriss and Burger, 1984; Burger and Hackett, 1986; Sim et al., 1989; Duran-Vila et al., 1992; Goh et al., 1995; Maggon and Singh, 1995; Pérez-Molphe-Balch and Ochoa-Alejo, 1997; Ghorbel et al., 1998; Garcia-Luis et al., 1999; Bordon et al., 2000; Moreira-Dias et al., 2000; Moreira-Dias et al., 2001), roots (Sauton et al., 1982; Burger e Hackett, 1986; Sim et al., 1989; Bhat et al., 1992; Gill et al., 1994; Goh et al., 1995), leaf sections (Chaturverdi and Mitra, 1974; Sim et al., 1989; Gill et al., 1994), and reproductive organs (Germana et al., 1994; Carimi et al., 1998; Perez et al., 1998). These previous studies have shown that responses to conditions of *in vitro* culture may vary according to genotype (Vardi and Spiegel-Roy, 1982; Carimi et al., 1998; Ghorbel et al., 1998; Perez et al., 1998; Bordon et al., 2000), explant type and orientation (Burger and Hackett, 1986; Sim et al., 1989; Gill et al., 1994; Goh et al., 1995; Maggon and Singh, 1995; Pérez-Molphe-Balch and Ochoa-Alejo, 1997; Perez et al., 1998; Garcia-Luis et al., 1999; Bordon et al., 2000; Moreira-Dias et al., 2000; Moreira-Dias et al., 2001), composition of the culture medium (Sauton et al., 1982; Burger and Hackett, 1986; Sim et al., 1989; Gill et al., 1994; Goh et al., 1995; Maggon and Singh, 1995; Pérez-Molphe-Balch and Ochoa-Alejo, 1997; Carimi et al., 1998; Chakravarty and

Goswami, 1999; Bordon et al., 2000; Moreira-Dias et al., 2000; Moreira-Dias et al., 2001), and conditions of incubation (Duran-Vila et al., 1992; Cabasson et al., 1997; Pérez-Molphe-Balch and Ochoa-Alejo, 1997; Perez et al., 1998; Garcia-Luis et al., 1999; Bordon et al., 2000; Moreira-Dias et al., 2000; Moreira-Dias et al., 2001). To our knowledge, not much attention have been given to variations arisen by differences on morphogenic potential of explants collected from different regions of the citrus epicotyl. In fact, there are just few and conflicting reports on the subject (Burger and Hackett, 1986; Sim et al., 1989; Goh et al., 1995; Garcia-Luis et al., 1999; Moreira-Dias et al., 2001). Burger and Hackett (1986) observed a decreasing on bud formation in both epicotyl and root sections from Valencia orange [*Citrus sinensis* (L.) Osb.] as the distance from the cotyledonary node increased. However, the expression of this gradient was remarkably modified by concentration of NAA in combination with BAP in the culture medium. A similar response was obtained by Moreira-Dias et al. (2001), who noted an inverse correlation between bud and shoot formation in epicotyl segments of Troyer citrange [*C. sinensis* (L.) Osb. x *Poncirus trifoliata* (L.) Raf.] and the distance of the segments from the cotyledonary node. However, the authors stated that the pattern of expression of the gradient was not affected by variations in the hormone concentration and lighting conditions, although these data are not explicit in the study. The same results described in Troyer citrange by Moreira-Dias et al. (2001) were also obtained by Garcia-Luis et al. (1999), but only when the epicotyl segments were placed vertically on the culture medium in an upright position. No consistent trend was observed by the authors for the effect of distance from cotyledonary node in explants placed horizontally or vertically on the culture medium in an inverted position. A completely opposite result was reported by Sim et al. (1989), who demonstrated that epicotyl segments of calamondin (*C. mitis* Blanco), collected from regions closer to the cotyledonary node, were less responsive than segments collected from more distant regions. Goh et al. (1995) also reported similar results in epicotyl segments from pummelo (*C. grandis* Osb.). These previous reports reveal that not much is known about the morphogenic gradient along the epicotyl axis of citrus and no systematic study has been undertaken aiming to understand possible factors affecting this process.

In the present report, we have compared *in vitro* bud and shoot regeneration from epicotyl explants of three citrus cultivars, not previously studied, and characterized in two of them the *in vitro* response of five distinct regions of the epicotyl to different growth regulator concentrations and conditions of incubation. Attempts have been made to identify particular regions of the epicotyl with higher morphogenic potential which could improve the efficiency of genetic transformation if used as source of explants.

MATERIALS AND METHODS

Plant materials

Etiolated seedlings of 'Cravo' rangpur lime (*Citrus limonia* Osb.), 'Foster' grapefruit (*C. paradisi* Macf.), and 'Pera' sweet-orange [*C. sinensis* (L) Osb.] were used as explant sources. After removing the coat, seeds were surface-sterilized for 2 minutes in 70% (v/v) ethanol, followed by immersion in a 2.5% (v/v) solution of sodium hypochlorite for 10 minutes, and then rinsed three times with sterile distilled water. The de-coated seeds were inoculated in 21 x 150 mm test tubes containing 10 ml of half-strength Murashige and Skoog (1962) medium with 25 g l^{-1} sucrose, 6 g l^{-1} agar (Sigma Chemical Co., USA), pH 5.7, and allowed to germinate at 27 ± 2^{o}C in the dark. In vitro-grown seedlings (5-10 cm long) of four different ages (21, 28, 35, or 42 days-old) were used as the source of epicotyl explants. To study the morphogenic response of different regions of the epicotyl (Fig. 1), each epicotyl was transversely cut into 1 cm long apical, sub-apical, median, sub-basal, and basal segments, and cultured on different culture media and conditions of incubation.

Culture medium and conditions of incubation

The inorganic salts of Murashige and Skoog (1962), supplemented with 100 mg l^{-1} inositol, 10 mg l^{-1} thiamine.HCl, 10 mg l^{-1} pyridoxine, 5 mg l^{-1} nicotinic acid, 25 g l^{-1} sucrose, and 8 g l^{-1} agar were used as the basal medium. The pH of the

medium was adjusted to 5.8 ± 0.1. The following concentrations of the growth regulator BAP were tested in the basal medium: 0, 0.5, 1.0, 2.0, 3.0, or 4.0 mg l^{-1}. The media were dispensed as 30-ml aliquots in disposable 9 cm sterile plastic Petri dishes (J. Prolab, Brazil) and sealed with Parafilm tape (American National Co., USA). The explants were placed horizontally in the media and incubated at 27 ± 2°C either in the 16/8-h light/dark regime (36 μmol m^{-2} s^{-1} light radiation) or in one of the four dark regimes (15, 21, 28 days, followed by exposition to 16/8-h light regime). After 45 days of incubation, regeneration frequency of the segments and number of shoots per explant were assessed. Regeneration frequency was calculated as the number of segments producing buds/shoots per total number of segments cultured.

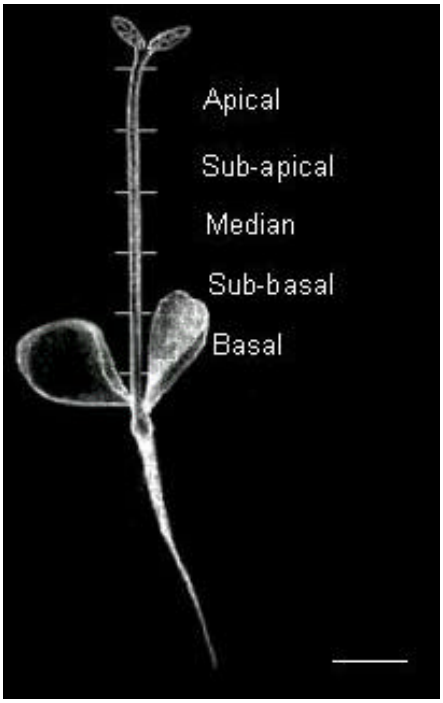


FIG. 1. Schematic representation of a 30 days-old citrus 'Foster' seedling showing the apical, sub-apical, median, sub-basal, and basal regions of the epicotyl (Bar = 10mm).

Experimental design and statistical analysis

Experiments were performed with 40 segments per treatment, provenient from 8 seedlings, and repeated twice. The five segments from each epicotyl were planted in the same Petri dish, allowing to determine the effect of the explant position on morphogenesis. Statistical analysis was performed with the software SAEG (Sistema de Análises Estatísticas e Genéticas, Universidade Federal de Viçosa, Brazil), which tested the experiments as a completely randomized design. Analysis of variance (ANOVA) was applied and for means comparison Tukey's test was employed, with a critical value of $P = 0.05$.

RESULTS

In vitro morphogenic responses in epicotyl segments to different concentrations of BAP

Since there are clear advantages in the selection of transgenic shoots when epicotyl explants are placed horizontally on the culture medium, this orientation was used in all experiments reported in the present study. The idea in this first set of experiments was to characterize the general *in vitro* responses of epicotyl explants from 'Cravo' rangpur lime, 'Foster' grapefruit, and 'Pera' sweet-orange to different concentrations of BAP, which have not been previously reported. The epicotyl explants of the three citrus cultivars analyzed followed the same pattern of *in vitro* morphogenesis in response to BAP concentrations. Both bud and shoot regeneration were inhibited by increasing the concentration of the growth regulator (Tables 1 and 2). From 2.0 mg l^{-1} BAP, epicotyl explants produced a higher callusing in the cut edges. On the surface of these calli, some adventitious buds differentiated through indirect organogenesis, but most failed to develop and no shoots were formed. At 1.0 mg l^{-1} BAP or lower concentrations, bud and shoot regeneration occurred in the surface of cut by direct organogenesis, without callus formation (Fig. 2B). In the absence of BAP (0 mg l^{-1}), suitable responses of regeneration were obtained in all citrus cultivars tested, mainly in terms of regeneration frequency. The optimal concentrations of BAP for bud and shoot regeneration in epicotyl explants were 0, 0.5, or 1.0 mg l^{-1}, irrespective of the genotype. In these concentrations of the growth regulator, there was a marked difference in the *in vitro* responses depending on

genotype. The better responses were obtained in ‘Foster’ grapefruit, followed by ‘Pera’ sweet-orange. The worse responses were obtained in ‘Cravo’ rangpur lime, that produced the lowest regeneration frequency values and number of shoots per explant.

Table 1- Influence of BAP concentration on the regeneration frequency from epicotyl explants of three citrus genotypes

Cultivar	BAP (mg l^{-1})					
	0	0.5	1.0	2.0	3.0	4.0
‘Cravo’	60.0 ± 9.1	32.5 ± 24	45.0 ± 31	15.0 ± 10	27.5 ± 22	16.7 ± 20
‘Foster’	87.5 ± 12	91.2 ± 9.5	90.8 ± 14	40.0 ± 16	41.2 ± 9.4	26.2 ± 11
‘Pera’	80.0 ± 29	70.0 ± 29	63.3 ± 35	20.0 ± 25	23.3 ± 25	46.7 ± 35

Data obtained from two independent experiments.
±. Standard deviation.

Table 2- Influence of BAP concentration on the number of shoots per explant of epicotyls from three citrus genotypes

Cultivar	BAP (mg l^{-1})					
	0	0.5	1.0	2.0	3.0	4.0
‘Cravo’	0.50 ± 0.2	0.47 ± 0.4	0.60 ± 0.5	0.10 ± 0.1	0.20 ± 0.2	0.13 ± 0.2
‘Foster’	1.05 ± 0.5	2.11 ± 1.1	2.47 ± 1.3	0.32 ± 0.3	0.23 ± 0.2	0.06 ± 0.1
‘Pera’	1.37 ± 0.7	2.12 ± 1.5	1.67 ± 1.1	0.47 ± 0.6	0.27 ± 0.3	0.60 ± 0.5

Data obtained from two independent experiments.
±. Standard deviation.

Effect of BAP concentration on the gradients of bud and shoot regeneration

To evaluate the morphogenic gradients in explants collected from different regions of the epicotyl, the citrus cultivars of best (‘Foster’ grapefruit) and worst (‘Cravo’ rangpur lime) morphogenic responses were chosen for further characterization. The general trend observed in both citrus cultivars was a higher regeneration frequency and higher number of shoots per explant as the distance of the

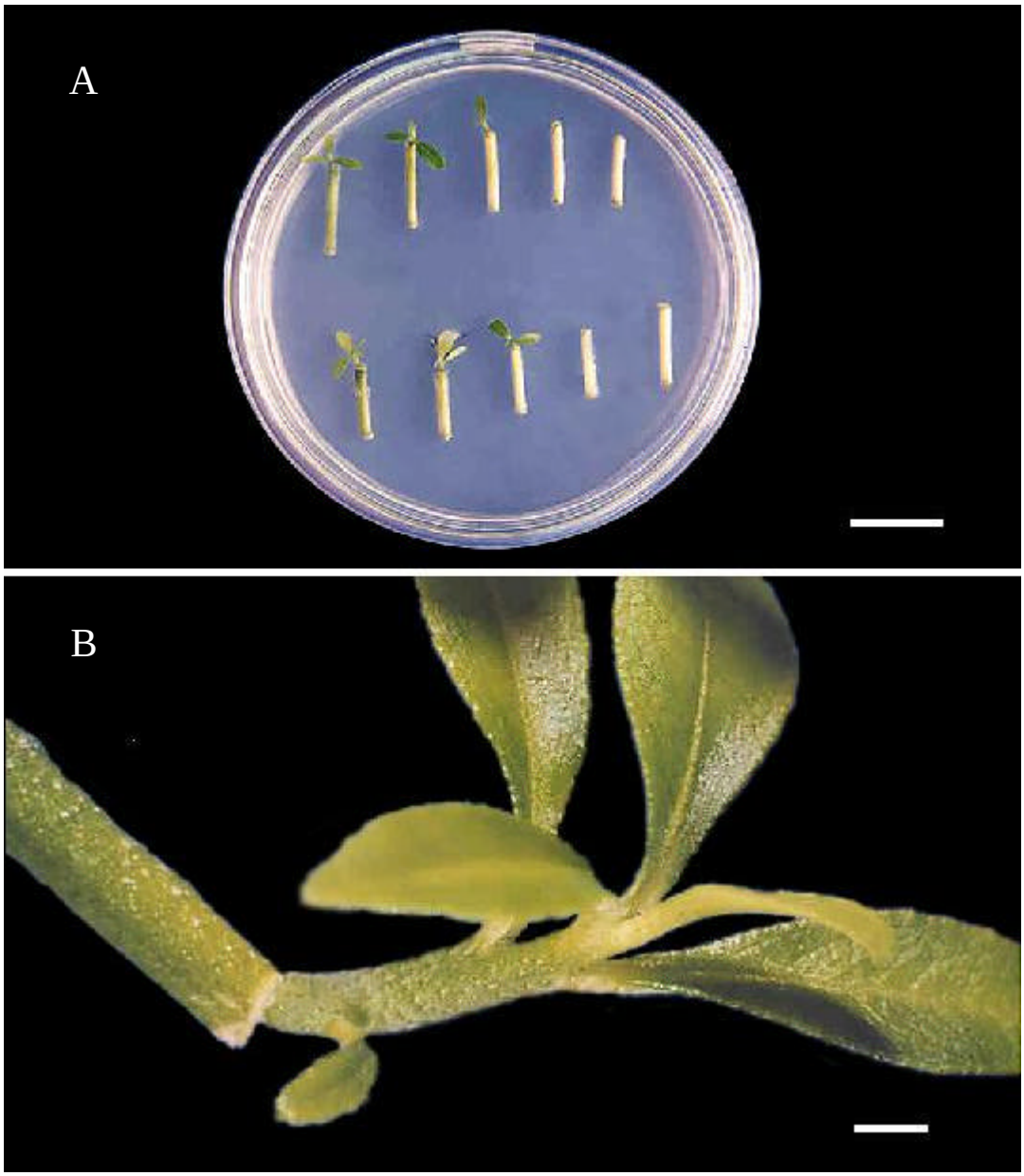


FIG. 2. *In vitro* morphogenesis of epicotyl explants from *Citrus*. **A -** Morphogenic gradient in epicotyl explants from 'Foster' grapefruit placed horizontally on culture medium containing 1.0 mg l^{-1} BAP and incubated in 16/8-h light regime. The distal end of each explant is at the top of the figure. Explants of the apical, sub-apical, median, sub-basal, and basal regions of the epicotyl are in sequence from left to the right of the figure (Bar = 1 mm); **B -** A detail on an adventitious shoot regenerated by direct organogenesis from ‘Cravo’ rangpur lime epicotyl explant placed horizontally on the culture medium containing 1.0 mg l^{-1} BAP (Bar = 10 mm). The pictures were taken 45 days after inoculation of the explants on culture medium.

epicotyl explants from the cotyledonary node increased, although these differences were not significant in every case. Changes in the concentration of BAP influenced the expression of this gradient along the epicotyl. In 'Cravo' rangpur lime, the gradient was expressed only in concentrations of 0.5 or 1.0 mg l^{-1} BAP, and no significant differences were observed among the different epicotyl regions in the absence (0 mg l^{-1}) or in 2.0 mg l^{-1} or in higher concentrations of BAP, as revealed by statistical analysis (Tables 3 and 4). A distinct response was obtained in 'Foster' grapefruit, in which the gradients in the regeneration frequency were significant only in the presence of 2.0 mg l^{-1} BAP (Table 3), but statistic difference in the number of shoots per explant was also observed in 1.0 mg l^{-1} BAP or lower concentrations (Table 4).

Effect of light regime on the gradients of bud and shoot regeneration

For this set of experiments, the concentration of 1.0 mg l^{-1} BAP was selected to be used in the culture medium. Light regime was also responsible for important effects in the expression of the morphogenic gradient along the epicotyl axis. It was observed in both citrus cultivars a higher callusing in the surface of cut when the explants were incubated in anyone dark regime, with adventitious shoots arising through indirect organogenesis. In 'Cravo' rangpur lime, the gradients on regeneration frequency were significant only by incubation of the epicotyl explants in the light or 21 days dark period (Table 5). Relevant differences on regeneration frequency between the different sections of the epicotyl were not observed when the explants were incubated in 15 or 28 days dark. A major effect of the light regime in the expression of the gradient in 'Cravo' was obtained in the number of shoots per explant (Table 6). Incubation of their explants in any period of dark led to insignificant difference on shoot regeneration along the different regions of the epicotyl axis. In 'Foster' grapefruit, changes in the light regime did not cause significant differences on regeneration frequencies between the different regions of the epicotyl (Table 5). However, a marked difference in this genotype was also observed for the number of shoots per explant (Table 6). The expression of the gradient was significant when the epicotyl explants were incubated in the light, 21 or

Table 3- Influence of BAP concentration on the regeneration frequency (%) in different regions of the citrus epicotyl

Genotype	Section	BAP (mg l^{-1})					
		0	0.5	1.0	2.0	3.0	4.0
'Cravo'	Apical	66.7 a	50.0 ab	75.0 a	25.0 a	50.0 a	50.0 a
	Sub-Apical	66.7 a	62.5 a	62.5 a	12.5 a	50.0 a	16.7 a
	Median	50.0 a	25.0 ab	62.5 a	12.5 a	0 a	16.7 a
	Sub-Basal	50.0 a	0 b	25.0 ab	0 a	12.5 a	0 a
	Basal	66.7 a	25.0 ab	0 b	25.0 a	25.0 a	0 a
'Foster'	Apical	100.0 a	100.0 a	100.0 a	68.7 a	50.0 a	43.7 a
	Sub-Apical	100.0 a	100.0 a	100.0 a	62.5 ab	43.7 a	25.0 a
	Median	87.5 a	81.2 a	100.0 a	31.2 bc	43.7 a	12.5 a
	Sub-Basal	75.0 a	93.7 a	87.5 a	50.0abc	25.0 a	25.0 a
	Basal	75.0 a	81.2 a	66.7 a	18.7 c	43.7 a	25.0 a

Data obtained from two independent experiments.
Means followed by the same letter are not statistically significant by Tukey's test ($P = 0.05$).

Table 4- Influence of BAP concentration on the number of shoots per explant in different regions of the citrus epicotyl

Genotype	Section	BAP (mg l^{-1})					
		0	0.5	1.0	2.0	3.0	4.0
'Cravo'	Apical	0.83 a	0.75 ab	1.37 a	0.25 a	0.62 a	0.50 a
	Sub-Apical	0.50 a	1.12 a	0.75 ab	0 a	0.25 a	0.17 a
	Median	0.50 a	0.12 b	0.75 ab	0 a	0 a	0 a
	Sub-Basal	0.33 a	0 b	0.12 b	0 a	0.12 a	0 a
	Basal	0.33 a	0.37 ab	0 b	0.25 a	0 a	0 a
'Foster'	Apical	1.68 a	3.62 a	3.96 a	0.87 a	0.56 a	0.06 a
	Sub-Apical	1.43 ab	2.93 a	3.50 a	0.37 a	0.18 a	0.18 a
	Median	1.06 ab	1.37 b	1.75 b	0.18 a	0.18 a	0.06 a
	Sub-Basal	0.62 ab	1.68 b	1.62 b	0.12 a	0 a	0 a
	Basal	0.50 b	1.00 b	1.16 b	0.06 a	0.25 a	0 a

Data obtained from two independent experiments.
Means followed by the same letter are not statistically significant by Tukey's test ($P = 0.05$).

Table 5- Influence of light regime on the regeneration frequency in different regions of the citrus epicotyl

Genotype	Section	Light regime			
		16/8-h light/dark	15 days dark	21 days dark	28 days dark
'Cravo'	Apical	75.0 a	43.7 a	68.7 a	56.2 a
	Sub-Apical	68.7 ab	43.7 a	50.0 ab	25.0 a
	Median	43.7 ab	56.2 a	37.5 ab	31.2 a
	Sub-Basal	25.0 b	37.5 a	31.2 ab	31.2 a
	Basal	31.2 ab	31.2 a	18.7 b	31.2 a
'Foster'	Apical	93.7 a	81.2 a	75.0 a	68.7 a
	Sub-Apical	81.2 a	75.0 a	100.0 a	100.0 a
	Median	68.7 a	75.0 a	100.0 a	100.0 a
	Sub-Basal	56.2 a	87.5 a	87.5 a	93.7 a
	Basal	56.2 a	68.7 a	87.5 a	81.2 a

Data obtained from two independent experiments.
Means followed by the same letter are not statistically significant by Tukey's test ($P = 0.05$).

Table 6- Influence of light regime on the number of shoots per explant in different regions of the citrus epicotyl

Genotype	Section	Light regime			
		16/8-h light/dark	15 days dark	21 days dark	28 days dark
'Cravo'	Apical	2.00 a	0.75 a	1.06 a	0.93 a
	Sub-Apical	1.43 a	0.62 a	0.93 a	0.50 a
	Median	1.93 a	1.12 a	0.75 a	0.81 a
	Sub-Basal	1.37 b	0.75 a	0.50 a	0.75 a
	Basal	0.87 b	0.81 a	0.43 a	0.81 a
'Foster'	Apical	2.81 a	2.37 a	1.50 b	1.50 b
	Sub-Apical	2.25 a	4.12 a	5.12 a	5.00 a
	Median	1.31 b	3.87 a	5.50 a	5.81 a
	Sub-Basal	0.81 b	3.31 a	5.06 a	5.25 a
	Basal	0.93 b	3.50 a	3.31 ab	2.93 ab

Data obtained from two independent experiments.
Means followed by the same letter are not statistically significant by Tukey's test ($P = 0.05$).

28 days dark (Fig. 2A). The incubation of the explants under 21 or 28 days dark was more dramatic, since they altered the general pattern of the gradient observed previously. Under these incubation conditions, there was a reduction on shoot regeneration in explants from apical and basal regions of the epicotyl, and an increase on shoot formation in explants from sub-apical, median, and sub-basal regions.

Effect of age of the seedling on the gradients of bud and shoot regeneration

In this study, the epicotyl explants were cultured on medium containing 1.0 mg l^{-1} BAP and incubated at 16/8-h light/dark regime. It was observed that the expression of the morphogenic gradient along the epicotyl axis was also affected by age of the seedlings. In 'Cravo' rangpur lime, significant differences on the regeneration frequencies in different regions of the epicotyl were obtained in seedlings from all ages evaluated (Table 7). However, when seedlings of 21 or 35 days old were used as source of epicotyl explants, the gradients in the number of shoots per explant were not significant in this genotype (Table 8). In 'Foster' grapefruit, only epicotyl explants from 28 days-old or older seedlings displayed significant differences in the gradient of regeneration frequency and number of shoots per explant (Tables 7 and 8). The expression of the gradient was not significant when epicotyl segments from younger seedlings (21 days old) were used as source of explant.

Table 7- Influence of age of the seedling on the regeneration frequency in different regions of the citrus epicotyl

Genotype	Section	Age (days)			
		21	28	35	42
'Cravo'	Apical	75.0 a	81.2 ab	87.5 a	56.2 a
	Sub-Apical	81.2 a	87.5 a	62.5 ab	62.5 a
	Median	43.7 ab	75.0 ab	62.5 ab	25.0 ab
	Sub-Basal	31.2 b	62.5 ab	37.5 b	6.2 b
	Basal	12.5 b	43.7 b	31.2 b	25.0 ab
'Foster'	Apical	87.5 a	100.0 a	75.0 ab	100.0 a
	Sub-Apical	100.0 a	100.0 a	100.0 a	100.0 a
	Median	62.5 a	100.0 a	75.0 ab	100.0 a
	Sub-Basal	100.0 a	62.5 ab	75.0 ab	50.0 b
	Basal	87.5 a	25.0 b	37.5 b	33.3 b

Data obtained from two independent experiments.
Means followed by the same letter are not statistically significant by Tukey's test ($P = 0.05$).

Table 8- Influence of age of the seedling on the number of shoots per explant in different regions of the citrus epicotyl

Genotype	Section	Age (days)			
		21	28	35	42
‘Cravo’	Apical	1.37 a	1.75 ab	1.81 a	1.31 ab
	Sub-Apical	1.18 a	2.25 a	1.37 a	1.56 a
	Median	0.56 a	1.18 ab	0.75 a	0.87 ab
	Sub-Basal	0.37 a	1.37 ab	0.56 a	0.06 b
	Basal	0.12 a	0.62 b	0.50 a	0.50 ab
‘Foster’	Apical	2.75 a	4.00 a	2.00 a	4.16 a
	Sub-Apical	2.87 a	3.50 a	2.12 a	3.16 ab
	Median	1.62 a	3.12 a	1.00 a	2.16 bc
	Sub-Basal	2.25 a	1.12 b	0.87 b	1.66 bc
	Basal	1.87 a	0.62 b	0.37 b	0.50 c

Data obtained from two independent experiments.
Means followed by the same letter are not statistically significant by Tukey’s test (*P = 0.05*).

DISCUSSION

We have characterized the *in vitro* responses of epicotyl explants from three citrus cultivars to different concentrations of BAP. To our knowledge, this is the first time that *in vitro* morphogenesis in epicotyl explants from 'Cravo' rangpur lime, 'Foster' grapefruit, and 'Pera' sweet-orange has been reported. BAP stimulated bud and shoot regeneration in an optimal concentration of 0.5-1.0 mg l^{-1}, irrespective of the genotype. Similar response to BAP, with a promotive effect in low concentration and inhibition at higher concentration, has also been described in the literature for explants from different citrus genotypes (Sim et al., 1989; Goh et al., 1995; Maggon and Singh, 1995).

The pathway of regeneration at the cut edges of the explants in the three cultivars tested was dependent on concentration of BAP. Epicotyl explants cultured on medium containing 1.0 mg l^{-1} BAP or lower concentrations produced shoots through a process of direct organogenesis. On the other hand, when 2.0 mg l^{-1} BAP or higher concentrations were used in the culture medium, shoots were produced by indirect organogenesis. Such response has apparently been observed in some citrus genotypes, whenever epicotyl explants are placed horizontally on the culture medium (Sim et al., 1989; Goh et al., 1995; Maggon and Singh, 1995). It has been reported recently that the explant orientation and polarity determine the regeneration pathway in epicotyl explants of citrus (Garcia-Luis et al., 1999), and that this morphogenic response is also influenced by genotype (Bordon et al., 2000) and conditions of incubation (Moreira-Dias et al., 2000; Moreira-Dias et al., 2001). Our results, also

supported by Sim et al. (1989), Goh et al. (1995), and Maggon and Singh (1995) observations, expand these findings and further demonstrate that the concentration of BAP determines the regeneration pathway in epicotyl explants placed horizontally on the culture medium.

The general pattern of the morphogenic gradient along the epicotyl axis observed in the present study was a higher organogenic response as the distance of the explants from cotyledonary node increased. However, the expression of this gradient was remarkably influenced by genotype, concentration of BAP, lighting conditions, and age of the seedlings, as revealed by statistical analysis. Even though the two citrus cultivars tested expressed the same pattern of the gradient, with maximal response nearest the apical region, the requirements for its expression were slightly different between them, as demonstrated in the Tables 3-8. These results confirm previous observations that the *in vitro* responses may vary according to citrus genotype (Vardi and Spiegel-Roy, 1982; Carimi et al., 1998; Ghorbel et al., 1998; Perez et al., 1998; Bordon et al., 2000).

BAP concentration was one of the most important factors affecting the morphogenic gradient along the epicotyl axis. The different regions of the citrus epicotyl shown distinct *in vitro* responses depending on concentration of the growth regulator. In low BAP concentrations (0.5-1.0 mg l^{-1}), there was a noticeable promotive effect on bud/shoot regeneration in epicotyl explants from apical, sub-apical, and median regions, and an inhibitory (‘Cravo’) or slight promotive (‘Foster’) effect on bud/shoot regeneration in epicotyl explants from sub-basal and basal regions (Tables 3 and 4). At 2.0 mg l^{-1} BAP or higher concentrations, explants from all regions of the epicotyl were sensitive to the inhibitory effect of this growth regulator. Such observation has already been described in similar experiments (Sim et al., 1989; Goh et al., 1995). Therefore, there are sufficient evidences that in explants placed horizontally on culture medium containing high levels of BAP, the non-expression of the morphogenic gradient along the epicotyl axis may be due to the toxic effect of BAP in explants from all regions of the epicotyl.

Light regime also had an important effect on the morphogenic gradient along the epicotyl axis. There were differences in the *in vitro* responses between the regions

of the citrus epicotyl under the different light regimes. However, the responses were genotype-specific. Whereas in ‘Cravo’ rangpur lime the incubation of explants in all dark regimes inhibited the *in vitro* morphogenesis, irrespective of the region of the epicotyl, in ‘Foster’ grapefruit the incubation in the dark stimulated bud/shoot regeneration in explants from basal, sub-basal, median, and sub-apical regions of the epicotyl and inhibited bud/shoot regeneration in explants from apical region. These results allow to conclude that dark regime tends to inhibit the expression of the morphogenic gradient along the epicotyl axis in ‘Cravo’ rangpur lime, and to inhibit or to alter the pattern of expression of the morphogenic gradient in ‘Foster’ grapefruit. Also, a major effect of the dark regime was the production of a significant callusing in the cut edges of the explants in both genotypes, changing the pathway of organogenesis from direct to indirect in culture medium containing 1.0 mg l^{-1} BAP. A similar result has been described in *C. sinensis* (Duran-Vila et al., 1992), and support previous investigations that light regime affects *in vitro* morphogenesis in epicotyl segments of citrus (Perez-Molphe-Balch and Ochoa-Alejo, 1997; Bordon et al., 2000; Moreira-Dias et al., 2000; Moreira-Dias et al., 2001).

The age of the seedlings was also important to expression of the morphogenic gradient in epicotyl of citrus, but did not alter the general pattern as did the light regime. The responses of the different regions of the epicotyl were also genotype-specific. In ‘Cravo’ rangpur lime, explants from all regions of the epicotyl were more responsive when seedlings of 28-35 days-old were used, with the order of higher response nearest of the apical region being maintained. In ‘Foster’ grapefruit, explants from the apical, sub-apical, and median regions of the epicotyl were more responsive as the age of the seedlings increased, whereas explants from the sub-basal and basal regions were more responsive in younger seedlings. Therefore, it is possible to infer that, at least for ‘Foster’ grapefruit, the use of older seedlings tend to accentuate the differences on morphogenic potential of the explants from different regions of the epicotyl, increasing the expression of the gradient along the epicotyl axis.

There are conflicting results in the literature about the pattern of expression of the morphogenic gradient along the epicotyl axis in citrus. Whereas some reports state that maximal response was obtained in regions nearest the cotyledons (Burger and

Hackett, 1986; Garcia-Luis et al., 1999; Moreira-Dias et al., 2001), others state that the opposite result was obtained (Sim et al., 1989; Goh et al., 1995). Some possibilities can be raised to explain these differences reported in the literature: (1) factors related to plant material (i.e., genotype, age of explants), (2) factors related to composition of the culture medium (i.e., type and concentration of growth regulators) or, (3) factors related to conditions of incubation (i.e., explant orientation, light regime). The first possibility, which represents some of the variations in the literature, could be ruled out since the pattern of expression of the morphogenic gradient, with maximal response nearest the apical regions, was not changed in the present study using distinct genotypes and age of the explants. The second possibility could be the main cause of the conflicting results reported in the literature. In the present study, the higher organogenic responses observed in explants from more apical regions, irrespective of the citrus genotype, were obtained using culture medium containing only BAP. Similar responses, with higher organogenic potential in explants nearest of apical regions, were also reported in *C. mitis* by Sim et al. (1989) and in *C. grandis* by Goh et al. (1995), both using only BAP as growth regulator in the culture medium. Otherwise, in the reports which opposite results were described, with maximal response nearest the cotyledons, the culture medium was supplemented by combinations of BAP and NAA (Burger and Hackett, 1986; Garcia-Luis et al., 1999; Moreira-Dias et al., 2001). It is known that BAP alone stimulates organogenesis at the distal end of cuttings, and the supplementation of NAA to BAP-containing culture medium is able to invert this morphogenic gradient (Skoog and Miller, 1957). The third possibility could have some minor effect on the pattern of expression of the morphogenic gradient. This is sustained by the fact that incubation of the explants from ‘Foster’ grapefruit in the dark changed the general pattern of expression of the gradient, with explants from apical region becoming less responsive. However, this was not true in ‘Cravo’ rangpur lime and also the divergent results reported in the literature were obtained under similar conditions of lighting (Burger and Hackett, 1986; Sim et al., 1989; Goh et al., 1995). A further result demonstrates that explant orientation affected the expression of the morphogenic gradient along the epicotyl

axis in ‘Troyer’ citrange, but did not alter the general pattern of expression, with higher response nearest the cotyledons (Garcia-Luis et al., 1999).

In conclusion, we have demonstrated that there is a morphogenic gradient along the epicotyl axis from ‘Cravo’ rangpur lime and ‘Foster’ grapefruit, with highest response nearest to apical region, and that this gradient was influenced by factors related to plant material, composition of the culture medium, and conditions of incubation. Since the apical, sub-apical, and median regions of the epicotyl from both genotypes produced the maximal *in vitro* responses, these could be used as source of explants in experiments of genetic transformation aiming to improve the efficiency of obtention of transgenic citrus plants.

Acknowledgements

We are indebted to Dr. Walter S.S. Filho (EMBRAPA-CNPMF, Cruz das Almas, Brazil) for kindly providing ‘Foster’ and ‘Pera’ seeds upon request. This research was supported by CAPES Foundation (Coordination of Higher Education and Graduate Training), Brasília/Brasil. MGC Costa is a recipient of a graduate fellowship from CAPES Foundation.

REFERENCES

Bhat, S. R.; Chitralekha, P.; Chandel, K. P. S. Regeneration of plants from long term root culture of lime, *Citrus aurantifolia* (Christm) Swing. Plant Cell, Tissue and Organ Culture 29:19-25; 1992.

Bordon, Y.; Guardiola, J. L.; Garcia-Luis, A. Genotype affects the morphogenic response *in vitro* of epicotyl segments of *Citrus* rootstocks. Annals of Botany 86:159-166; 2000.

Burger, D. W.; Hackett, W. P. Gradients of adventitious bud formation on excised epicotyl and root sections of citrus. Plant Science 43:229-232; 1986.

Cabasson, C.; Alvard, D.; Dambier, D.; Ollitrault, P.; Teisson, C. Improvement of *Citrus* somatic embryo development by temporary immersion. Plant Cell, Tissue and Organ Culture 50:33-37; 1997.

Carimi, F.; Tortorici, M. C.; De Pasquale, F.; Crescimanno, F. G. Somatic embryogenesis and plant regeneration from undeveloped ovules and stigma/style explants of sweet orange navel group [*Citrus sinensis* (L.) Osb.]. Plant Cell, Tissue and Organ Culture 54:183-189; 1998.

Chakravarty, B.; Goswami, B. C. Plantlet regeneration from long-term callus cultures of *Citrus acida* Roxb. and the uniformity of regenerated plants. Scientia Horticulturae 82:159-169; 1999.

Chaturvedi, H. C.; Mitra, G. Clonal propagation of citrus from somatic callus culture. HortScience 9:118-120; 1974.

Duran-Vila, N.; Gogorcena, Y.; Ortega, V.; Ortiz, J.; Navarro, L. Morphogenesis and tissue culture of sweet orange (*Citrus sinensis* (L) Osb): effect of temperature and photosynthetic radiation. Plant Cell, Tissue and Organ Culture 29:11-18; 1992.

Edriss, M. H.; Burger, D. W. *In vitro* propagation of Troyer citrange from epicotyl segments. Scientia Horticulturae 23:159-162; 1984.

Garcia-Luis, A.; Bordon, Y.; Moreira-Dias, J. M.; Molina, R. V.; Guardiola, J. L. Explant orientation and polarity determine the morphogenic response of epicotyl segments of Troyer citrange. Annals of Botany 84:715-723; 1999.

Germana, M. A.; Wang, Y. Y.; Barbagallo, M. G.; Iannolino, G.; Crescimanno, F. G. Recovery of haploid and diploid plantlets from anther culture of *Citrus clementina* Hort. ex Tan. and *Citrus reticulata* Blanco. Journal of Horticultural Science 69:473-480; 1994.

Ghorbel, R.; Navarro, L.; Duran-Vila, N. Morphogenesis and regeneration of whole plants of grapefruit (*Citrus paradisi*), sour orange (*C. aurantium*) and alemow (*C. macrophylla*). Journal of Horticultural Science & Biotechnology 73:323-327; 1998.

Gill, M. I. S.; Singh, Z.; Dhillon, B. S.; Gosal, S. S. Somatic embryogenesis and plantlet regeneration on calluses derived from seedling explants of Kinnow mandarine (*Citrus nobilis* Lour x *Citrus deliciosa* Tenora). Journal of Horticultural Science 69:231-236; 1994.

Goh, C. J.; Sim, G. E.; Morales, C. L.; Loh, C. S. Plantlet regeneration through different morphogenic pathways in pommelo tissue culture. Plant Cell, Tissue and Organ Culture 43:301-303; 1995.

Grinblat, V. Differentiation of citrus stem *in vitro*. Journal of the American Society for Horticultural Science 97:599-603; 1972.

Maggon, R.; Singh, B. D. Promotion of adventitious bud regeneration by ABA in combination with BAP in epicotyl and hypocotyl explants of sweet orange (*Citrus sinensis* L. Osbeck). Scientia Horticulturae 63:123-128; 1995.

Moreira-Dias, J. M.; Molina, R. V.; Bordon, Y.; Guardiola, J. L.; Garcia-Luis, A. Direct and indirect shoot organogenic pathways in epicotyl cuttings of Troyer

citrange differ in hormone requirements and in their response to light. Annals of Botany 85:103-110; 2000.

Moreira-Dias, J. M.; Molina, R. V.; Guardiola, J. L.; Garcia-Luis, A. Daylength and photon flux density influence the growth regulator effects on morphogenesis in epicotyl segments of Troyer citrange. Scientia Horticulturae 87:275-290; 2001.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum 13:473-497; 1962.

Perez, R. M.; Galiana, A. M.; Navarro, L.; Duran-Vila, N. Embryogenesis *in vitro* of several *Citrus* species and cultivars. Journal of Horticultural Science & Biotechnology 73:796-802; 1998.

Pérez-Molphe-Balch, E.; Ochoa-Alejo, N. *In vitro* plant regeneration of Mexican lime and mandarin by direct organogenesis. HortScience 32:931-934; 1997.

Sauton, A.; Mouras, A.; Lutz, A. Plant regeneration from citrus root meristems. Journal of Horticultural Science 57:227-231; 1982.

Sim, G. E.; Goh, C. J.; Loh, C. S. Micropropagation of *Citrus mitis* Blanco – multiple bud formation from shoot and root explants in the presence of 6-benzylaminopurine. Plant Science 59:203-210; 1989.

Skoog, F.; Miller, C.O. Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symposium of Society for Experimental Biology 11:118-131; 1957.

Vardi, A.; Spiegel-Roy, P.; Galun, E. Plant regeneration from citrus protoplasts: variability in methodological requirements among cultivars and species. Theoretical and Applied Genetics 62:171-176; 1982.

CAPÍTULO III

AN EVALUATION OF FACTORS AFFECTING THE EFFICIENCY OF *Agrobacterium*-MEDIATED TRANSFORMATION OF *Citrus paradisi* (Macf.) AND OBTENTION OF TRANSGENIC PLANTS CONTAINING CAROTENOID BIOSYNTHETIC GENES UNDER CONSTITUTIVE EXPRESSION

ABSTRACT

An improved protocol for *Agrobacterium*-mediated transformation of 'Duncan' grapefruit (*Citrus paradisi* Macf.) epicotyl explants was developed by examining the effects of six different factors on the efficiency of transformation and combining the best treatments for each factor. The preculturing of explants and the composition of the cocultivation medium were the factors that most influenced transformation efficiency. The optimized protocol was successfully employed in the production of transgenic grapefruit plants containing the carotenoid biosynthetic genes phytoene synthase, phytoene desaturase, or lycopene-β-cyclase under constitutive expression. With an eventual goal of metabolically engineering grapefruit with multiple genes, hygromycin as a selectable marker and BIBAC as a transformation vector for large pieces of DNA were also tested.

Key words *Citrus paradisi* · grapefruit · genetic transformation · carotenoids

Abbreviations *BAP* 6-benzylaminopurine · *2,4-D* 2,4-dichlorophenoxyacetic acid · *NAA* naphtaleneacetic acid · *GUS* β-glucuronidase · *nptII* neomycin phosphotransferase II · *hptII* hygromycin phosphotransferase II · *cDNA* complementary DNA

INTRODUCTION

A decade has passed since the first report of *Agrobacterium*-mediated transformation of stem segments and regeneration of transgenic citrus plants (Moore et al. 1992). Since then, protocols for *Agrobacterium*-mediated transformation have been published for a number of citrus and related species (Kaneyoshi et al. 1994, Peña et al. 1995a, Peña et al. 1995b, Gutiérrez-E et al. 1997, Peña et al. 1997, Bond and Roose 1998, Cervera et al. 1998, Pérez-Molphe-Balch and Ochoa-Alejo 1998, Luth and Moore 1999, Cervera et al. 2000, Domínguez et al. 2000, Yang et al. 2000, Peña et al 2001, Ghorbel et al. 2001). However, citrus transformation is still relatively inefficient for some citrus types. The objective of the present study was to evaluate the influence of six different factors on the transformation efficiency of 'Duncan' grapefruit (*Citrus paradisi* Macf.), aiming to develop an optimized protocol to be used in the incorporation of the carotenoid biosynthetic genes phytoene synthase, phytoene desaturase, or lycopene-β-cyclase. *C. paradisi* is of special interest because variations in the carotenoid biosynthetic pathway can be readily observed by the accumulation of various carotenoid biosynthetic intermediates in the white, pink, and red fleshed cultivars. Also, the accumulation of a high content of naringin, a flavonoid compound involved in the bitter taste of the fruits, makes grapefruit a good citrus species to be used in metabolic engineering aiming to manipulate provitamin *A* composition, color, and flavor of the fruit and juice. Because metabolic engineering may require the addition of several transgenes (Ye et al

2000), the use of hygromycin as a selective agent and the use of the BIBAC vector plasmid for insertion of large pieces of DNA (Hamilton 1997) were also evaluated.

MATERIALS AND METHODS

Plant material and *Agrobacterium* transformation

Preparation of epicotyl explants from etiolated seedlings of 'Duncan' grapefruit, *Agrobacterium* transformation, scoring regenerated shoots for β-glucuronidase (*GUS*) expression, and rooting of shoots were as described in Luth and Moore (1999).

Agrobacterium strains and plasmids

The disarmed *Agrobacterium tumefaciens* strain EHA-101 (Hood et al. 1986) harboring vector plasmid pGA482GG (Slightom 1991) was employed in experiments to improve the protocol (Gutiérrez-E. et al. 1997) (Figure 1). For the experiments testing hygromycin as a selective agent, EHA-105 (Hood et al. 1993) containing either pCAMBIA 1301 or pCAMBIA 2301 (www.cambia.org.au) was used; the plasmids are identical except for the presence of the chimeric genes hygromycin phosphotransferase II (*hptII*) and neomycin phosphotransferase II (*nptII*), respectively (Figure 1). For transformation with carotenoid biosynthetic genes, three constructs were engineered: the cDNAs encoding phytoene synthase, under control of the CaMV 35S promoter, phytoene desaturase, under control of the figwort mosaic virus 34S (FMV 34S) promoter, and lycopene-β-cyclase, under control of the CaMV 35S promoter, were inserted individually into the *PstI* site of the pCAMBIA

2301 plasmid to generate pCAMBIA 2301/CitPSY, pCAMBIA 2301/CitPDS, and pCAMBIA 2301/CitLCY-B, respectively. The full length cDNA sequences of these genes were identified, cloned and characterized from fruits of *C. paradisi* cv. 'Flame', and have been deposited in the GenBank Database of the National Center for Biotechnology Information (NCBI) under the access numbers AF152892 (CitPSY), AF364515 (CitPDS), and AF152246 (CitLCY-B) (Costa 2002, unpublished results). The plasmids were introduced into the EHA-105 *Agrobacterium* strain by direct DNA uptake (An 1987).

Factors evaluated

Six factors were evaluated for their effects on transformation efficiency. Different treatments were compared for each factor: (1) the effect of preculture of explants on cocultivation medium; (2) length of incubation of explants in *Agrobacterium* solution; (3) concentration of *Agrobacterium* in the solution; (4) effect of 2,4-D in the cocultivation medium; (5) length of cocultivation period, and; (6) optimum BAP concentration in the selective regeneration medium.

Hygromycin as a selective agent

Explant segments were first cultured on regeneration medium containing different levels of hygromycin to identify potential concentrations for selecting transformed shoots. Explants were then cultured on selective regeneration medium supplemented with either hygromycin (after inoculation with pCAMBIA 1301) and 250 mg L^{-1} claforan or 75 mg L^{-1} kanamycin (pCAMBIA 2301) and 250 mg L^{-1} claforan, in order to compare side-by-side the transformation efficiency of several dosages of hygromycin with the control kanamycin.

BIBAC as a transformation vector

The transformation efficiency of BIBAC (Hamilton, 1997) was assessed using two constructs: BIBAC2 empty vector (UIA-143 pMP90 pCH32 BiBAC2) and BIBAC2.H150 (UIA-143 pMP90 pCH32 BiBAC2.H150) containing a 150

kb human genomic clone (Figure 1). The vector plasmids were in *A. tumefaciens* strain UIA 143 pMP90 harboring the virulence helper plasmid pCH32, which overexpresses the *VirG* and *VirE2,* virulence proteins involved in transcriptional activation of *vir* genes and protection of the T-DNA from degradation by endonucleases, respectively. Since the *Agrobacterium* strain that contains the BIBAC2 empty vector has the active *sacB* gene that makes bacterial cells sensitive to high levels of sucrose, this carbon source was replaced by glucose in the cocultivation and selective regeneration media. For the *Agrobacterium* construct containing BIBAC2.H150, the regular culture media were used, since the *sacB* gene has been inactivated.

Experimental design

Approximately 70 explants (5 plates) were used for each treatment and the data from all plates of a particular treatment were pooled. Each experiment was repeated at least once and, in some cases, twice. Transformation efficiency was evaluated after 45 days on selective regeneration medium based on the numbers and percentages of GUS positive (GUS^+) shoots produced. Shoots that arose from the same explant were considered independent transformation events.

Polymerase chain reaction (PCR) analysis

Plant DNA was isolated using the DNAzol® method described by Chomczynski et al. (1997). PCR was carried out in 50-µl volumes containing 0.2 m*M* of each dNTP, 0.25 µ*M* of each oligonucleotide primer, 0.25 U *Taq* polymerase and 100 ng of sample DNA. The reactions were amplified in a thermal cycler (MJ Research) with: an initial denaturing at 94°C for 2 min; 35 cycles of 94 °C for 1 min, 63°C for *nptII* or 53°C for *gus* for 1 min, 72°C for 2 min; and a final extension at 72°C for 10 min. Amplified DNA fragments were electrophoresed on a 2% agarose gel, stained with ethidium bromide and visualized under UV light. The primers used for amplification of the fragment of the *nptII* gene were 5'-ATGGGGATTGAACAAGATGGATTG-3' and 5'-TCA-

GAAGAACTCGTCAAGAAGGC-3', generating an 800-bp product; those used for amplification of the fragment of the *gus* gene were 5'-CAACGAACTGAAC-TGGCAG-3' and 5'-CATCACCACGCTTGGGTG-3', generating a 800 bp product.

Southern hybridization

A Southern blot was prepared by digesting 10 μg genomic DNA with *Hpa*I, separating the DNA fragments on a 1% agarose gel and transferring them to a positively charged nylon membrane (Boehringer Mannheim). The membrane was hybridized with a digoxigenin-11-dUTP-labeled probe for GUS, which was generated by amplifying a 800 bp DNA fragment from the GUS gene of pGA482GG. Hybridization was carried out following the DIG Application Manual (Roche) in DIG Easy Hyb buffer overnight at 42^{o}C, using 2 μl probe per ml hybridization solution. After the membrane washings, hybridized DNA bands were visualized following the DIG chemiluminescent detection protocol provided by the manufacturer (Roche). X-ray films were exposed for 10 min.

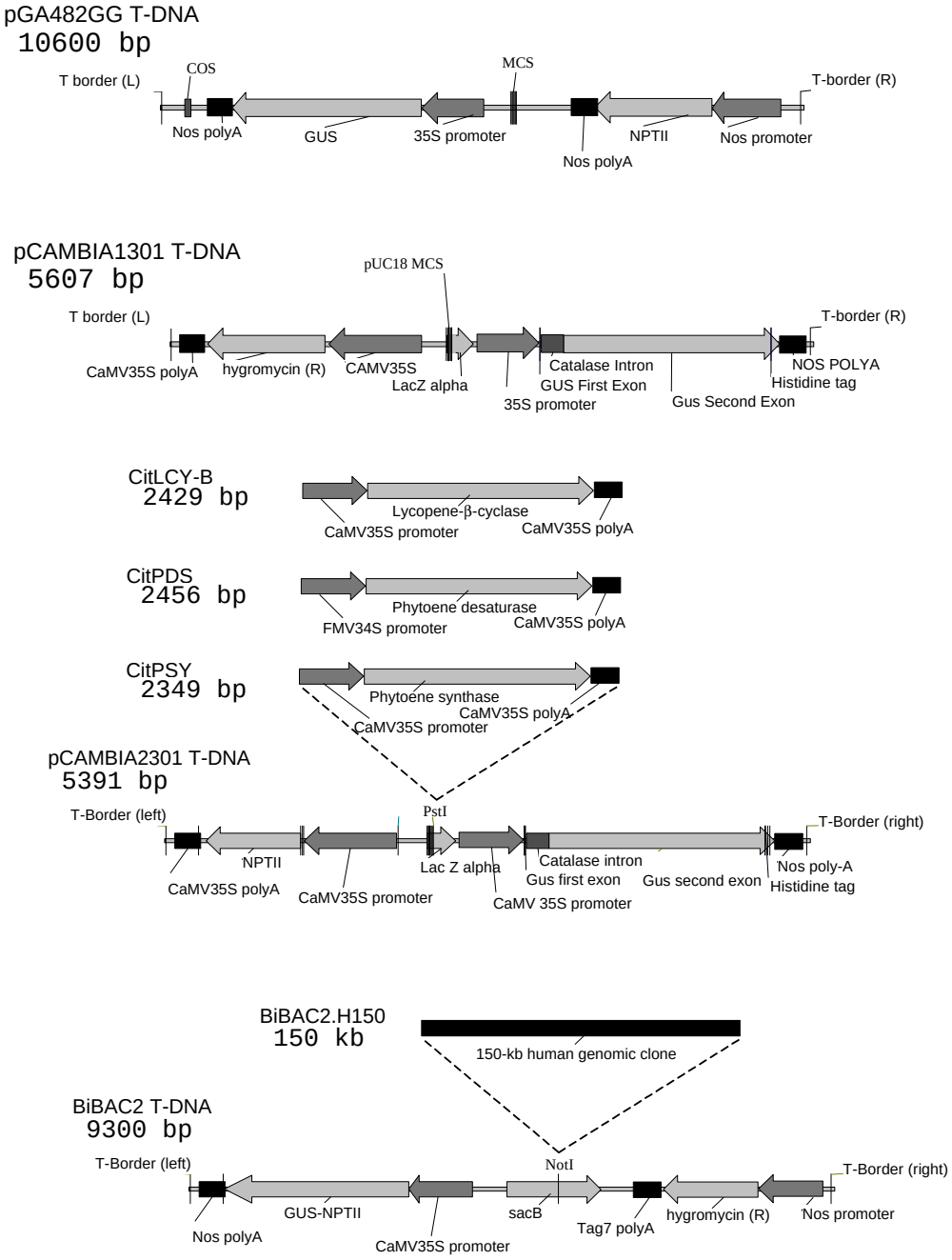


Figure 1- Schematic representation of the T-DNA region from the plasmids used in the experiments of genetic transformation.

RESULTS AND DISCUSSION

Analysis of the factors affecting the transformation efficiency of 'Duncan' grapefruit

The group of Peña et al. have published a protocol for transformation of citrus epicotyl segments that differs from ours in several details. In particular, explants are kept in darkness for the first 4 weeks of selection, fostering callus production (Cervera et al. 1998). The explants are then moved into light for regeneration of transgenic shoots from callus. In our protocol, explants are maintained in light throughout the selection period and shoots arise directly from the explant without an intervening callus phase (Luth and Moore 1999). We wished to retain that part of our protocol because it is rapid and less likely to lead to somaclonal variation. Therefore we evaluated other factors that Cervera et al. (1998) found to influence transformation rates.

Preculturing

Preculture of 'Duncan' epicotyl segments on cocultivation medium prior to incubation in *Agrobacterium* solution drastically reduced the numbers and percentages of segments producing shoots, total shoots produced, total GUS^+ shoots, and segments with GUS^+ shoots (Table 1). The percentage of segments with GUS^+ shoots, which is considered one of the most informative definitions of genetic transformation efficiency, was 8.4-fold higher in the treatment without preculture (5.9%) compared to both 1- and 2-day preculture treatments (0.7%). A

similar but less dramatic result was described in citrus by Cervera et al. (1998), who noted that 1-day preculture of 'Carrizo' citrange stem segments on cocultivation medium reduced the number of GUS^+ spots on explants to less than half the number obtained without preculture.

The basis of the promotion of *Agrobacterium*-mediated transformation by preculturing explants is not completely established, but it has been proposed that the production of *vir*-inducing compounds by metabolically active cells plays an important role (Stachel et al. 1985, Spencer and Towers 1991). In fact, Sunilkumar et al. (1999) demonstrated that the preculturing step in the transformation of tobacco leaf discs could be bypassed by providing acetosyringone to the freshly cut explants. In our experiments, acetosyringone was supplied to freshly cut epicotyl explants in both the cocultivation medium and the *Agrobacterium* resuspension, irrespective of the treatment. Thus, a promotive effect of preculturing on transformation efficiency may not have been observed due to the presence already of acetosyringone and the negative effect of preculturing could be attributed to problems caused to the physiology of the explants.

Incubation period

In general, explants incubated for 5-, 10-, or 20-min in *Agrobacterium* solution produced more shoots than explants incubated for 1-min (Table 1). However, many of these shoots were escapes (shoots that regenerated but were not GUS+). In citrus, a range of from 10- to 30-min incubation of the explants in *Agrobacterium* resuspension has been employed (Kaneyoshi et al. 1994, Peña et al. 1997, Bond and Roose 1998, Cervera et al. 1998, Luth and Moore 1999, Yang et al. 2000, Ghorbel et al. 2001), but no experiments comparing different incubation periods side-by-side have been reported. However, Bond and Roose (1998) observed that any incubation period significantly longer than 10-min appeared to decrease the transformation efficiency of 'Washington' navel orange [*C. sinensis* (L.) Osb.] epicotyl explants. We saw this trend only in that longer incubation periods led to the presence of more escape shoots. This is not

troublesome when GUS-containing vector plasmids are used, as the GUS assay is simple to perform. Therefore, in the absence of any other clear-cut trend, a 20-min cocultivation period was selected for use in further experiments since slightly more total GUS^+ shoots were produced in this treatment.

Agrobacterium concentration

The number of *Agrobacterium* cells in the inoculum is considered a critical factor in the efficiency of transformation; an excessive number of bacteria can stress plant cells and affect their regeneration potential, whereas low concentrations can reduce the frequency of T-DNA transfer. In citrus, $5x10^8$ cfu ml^{-1} has been used in some genetic transformation experiments (Kaneyoshi et al. 1994, Bond and Roose 1998, Luth and Moore 1999, Yang et al. 2000), while $4x10^7$ cfu ml^{-1} has been used in others (Peña et al. 1995a, Peña et al. 1995b, Peña et al. 1997, Cervera et al. 1998, Domínguez et al. 2000, Ghorbel et al., 2001), representing a 12.5-fold lower inoculum number. Peña et al. (1995b), comparing the *Agrobacterium* concentrations of $4x10^7$ and $4x10^8$ cfu ml^{-1} on transformation efficiency of 'Carrizo' citrange stem segments, obtained the best transformation rates using the former concentration. In the present experiment, no shoots were produced when epicotyl explants were inoculated in $2x10^8$ cfu ml^{-1} *Agrobacterium*. However, very few shoots were obtained in any of the treatments; therefore no conclusions can be drawn.

Cocultivation medium

It has been reported that certain auxins, especially 2,4-D, increase transformation frequencies when added to the cocultivation and/or preculturing medium. In citrus, the group of Peña et al obtained improved transformation frequencies by using tomato cell feeder layers on a medium high in auxins during the cocultivation period (Peña et al. 1997, Cervera et al. 1998, Domínguez et al. 2000). Upon evaluating separately the effects of the feeder plate medium, the filter paper layer, and the tomato cell suspensions, the best transformation

frequency was obtained with the auxin-rich medium alone (Cervera et al. 1998). Therefore, we performed an experiment to investigate the effect of 2,4-D in the cocultivation medium in our transformation system. Surprisingly, the addition of this growth regulator to the cocultivation medium was responsible for higher regeneration and transformation frequencies, in an optimal range from 0.2 to 1.0 mg L^{-1} (Table 1). Even though the epicotyl explants were exposed to auxin for only 2 days, there was more callusing on their cut edges than in the control, and the callusing increased as the concentration of the growth regulator increased. In explants cultured on cocultivation medium containing 2.0 mg L^{-1} 2,4-D, some adventitious buds differentiated on the surface of the calli through indirect organogenesis, but most failed to develop further and few shoots were formed. The maximum number of segments producing shoots, total number of shoots produced, number of GUS^+ shoots, and number of segments with GUS^+ shoots was obtained in 0.2 mg L^{-1} 2,4-D.

Cocultivation period

Cervera et al. (1998) observed a higher transient transformation frequency of 'Carrizo' citrange stem segments as the cocultivation period increased, reaching a maximum at 5-days, but this cocultivation length also resulted in abundant proliferation of bacteria and the consequent decrease in the regeneration frequency of transformed shoots. Here, when explants were transferred to selective regeneration medium after a 1-day cocultivation period, few shoots were obtained and no transformation was observed. Higher numbers of segments producing shoots were obtained when the explants were cocultured with *Agrobacterium* for a period of 3-4 days (Table 1), but decreased numbers of GUS^+ shoots were obtained. The highest numbers of GUS^+ shoots and segments with GUS^+ shoots were obtained with a 2-day cocultivation period.

BAP concentration in selective regeneration medium

We previously demonstrated that relatively high concentrations of BAP in selective regeneration medium, although necessary for shooting, reduced the ability of regenerated shoots to subsequently root (Gutiérrez-E. et al. 1997). For this reason, we have routinely used a medium containing 0.5-1.0 mg L^{-1} BAP, but the optimum level for grapefruit has not been determined. In the present experiments, the largest numbers of segments producing shoots were obtained at concentrations of 1.0-2.0 mg L^{-1} BAP, and reduced numbers of shoots were observed in the absence of (0 mg L^{-1}) or at higher concentrations of (3.0-4.0 mg L^{-1}) BAP (Table 1). The highest numbers of GUS^{+} shoots were found at concentrations of 0.5-2.0 mg L^{-1} BAP, whereas no GUS^{+} shoots were produced in the absence of BAP. Thus, the best transformation efficiency, represented by the number of segments with GUS^{+} shoots, was obtained on 2.0 mg L^{-1} BAP (6.1%), which increased the transformation rate from 1.3- to 2.6-fold compared to medium containing 1.0 and 0.5 mg L^{-1} BAP, respectively. Since we have demonstrated that BAP concentrations of 2.5 mg L^{-1} or less in the regeneration medium did not inhibit the subsequent rooting of the shoots produced (Gutiérrez-E. et al. 1997), 2.0 mg L^{-1} was chosen as the most suitable BAP concentration to be used in the selective regeneration medium of ‘Duncan’ grapefruit epicotyl explants. Moreover, we have observed that the presence of 2,4-D in cocultivation medium increased the rooting efficiency of shoots regenerated in selective medium containing this level of BAP (results not shown).

Table 1- Effect of different factors affecting the transformation efficiency of 'Duncan' grapefruit (*C. paradisi* Macf.)

Factor evaluated	Treatment[a]	Number of segments cultured[b]	Number (%) of segments producing shoots	Total number shoots produced	Number (%) of GUS^+ shoots	Number (%) of segments with GUS^+ shoots
Preculturing	without preculture	136	11 (8.1)	11	8 (72.7)	8 (5.9)
	1 day	142	3 (2.1)	3	1 (33.3)	1 (0.7)
	2 days	132	6 (4.5)	6	1 (16.7)	1 (0.7)
Incubation period in *Agrobacterium*	1 minute	135	11 (8.1)	11	6 (54.5)	6 (4.4)
	5 minutes	130	22 (16.9)	22	7 (31.8)	7 (5.4)
	10 minutes	139	16 (11.5)	17	7 (41.2)	7 (5.0)
	20 minutes	132	17 (12.9)	21	9 (42.8)	9 (6.8)
Agrobacterium concentration	2×10^8 cfu ml^{-1}	120	0	0	0	0
	3×10^8 cfu ml^{-1}	131	3 (2.3)	3	3 (100)	3 (2.3)
	4×10^8 cfu ml^{-1}	136	2 (1.5)	2	1 (50)	1 (0.7)
	5×10^8 cfu ml^{-1}	145	3 (2.1)	3	3 (100)	3 (2.1)
Cocultivation medium	0 mg L^{-1} 2,4-D	142	7 (4.9)	8	2 (25.0)	2 (1.4)
	0.2 mg L^{-1} 2,4-D	142	23 (16.2)	27	12 (44.4)	12 (8.4)
	0.5 mg L^{-1} 2,4-D	140	19 (13.6)	21	5 (23.8)	5 (3.6)
	1.0 mg L^{-1} 2,4-D	138	12 (8.7)	13	1 (7.7)	1 (0.7)
	2.0 mg L^{-1} 2,4-D	137	3 (2.2)	3	1 (33.3)	1 (0.7)
Cocultivation period	1 day	137	2 (1.4)	3	0	0
	2 days	138	13 (9.4)	15	7 (46.7)	7 (5.0)
	3 days	140	17 (12.1)	17	6 (35.3)	6 (4.3)
	4 days	139	18 (12.9)	18	2 (11.1)	2 (1.4)
BAP concentration in selective medium	0 mg L^{-1}	127	5 (3.9)	5	0	0
	0.5 mg L^{-1}	128	7 (5.5)	13	6 (46.1)	3 (2.3)
	1.0 mg L^{-1}	127	13 (10.2)	15	6 (40.0)	6 (4.7)
	2.0 mg L^{-1}	132	14 (10.6)	19	8 (42.1)	8 (6.1)
	3.0 mg L^{-1}	128	6 (4.7)	8	3 (37.5)	3 (2.3)
	4.0 mg L^{-1}	127	6 (4.7)	8	2 (25.0)	2 (1.6)

[a]Explants inoculated with EHA-101::pGA482GG.
[b]Data are the summary of two independent experiments.

Improved protocol

The results from these studies were combined to produced an enhanced protocol for the transformation of grapefruit. The protocol includes incubation of the explants without preculturing for 20-min in *Agrobacterium* solution at $5x10^8$ cfu ml^{-1}, followed by a 2-day cocultivation period on cocultivation medium supplemented with 0.2 mg L^{-1} 2,4-D, and then transfer of the explants to selective regeneration medium containing 2.0 mg L^{-1} BAP. Further experiments demonstrated a higher transformation efficiency for 'Duncan' grapefruit using this protocol than had been obtained previously (results not shown). To recover the transgenic plants, GUS-positive shoots were rooted in culture medium as previously described by Luth and Moore (1999). A rooting frequency of 90% was observed. Eleven GUS-positive shoots were tested with PCR for the presence of the introduced gene. All of these shoots showed the predicted band for the GUS gene (Figure 2a), even though the bands in lanes 1, 4, 7, and 10 were very weak. Integration of the T-DNA containing the GUS gene and its copy number was confirmed by Southern hybridization analysis (Figure 2b). Digestion with *Hpa*I and hybridization with the GUS probe resulted in bands larger than 6 kb for most putatively transgenic plants. These bands were expected to contain not only the GUS gene and the left T-DNA border but also a plant DNA fragment that varied in size depending on the insertion site. Between one and three bands were detected in the different transgenic plants, suggesting that at least one to three copies of the GUS gene were integrated (The bands in lane 7 were very faint). One plant did not show any hybridization signal (Fig. 2b, lane 3), which provides evidence for partial T-DNA integration. No hybridization bands were present in the untransformed shoots (lane C).

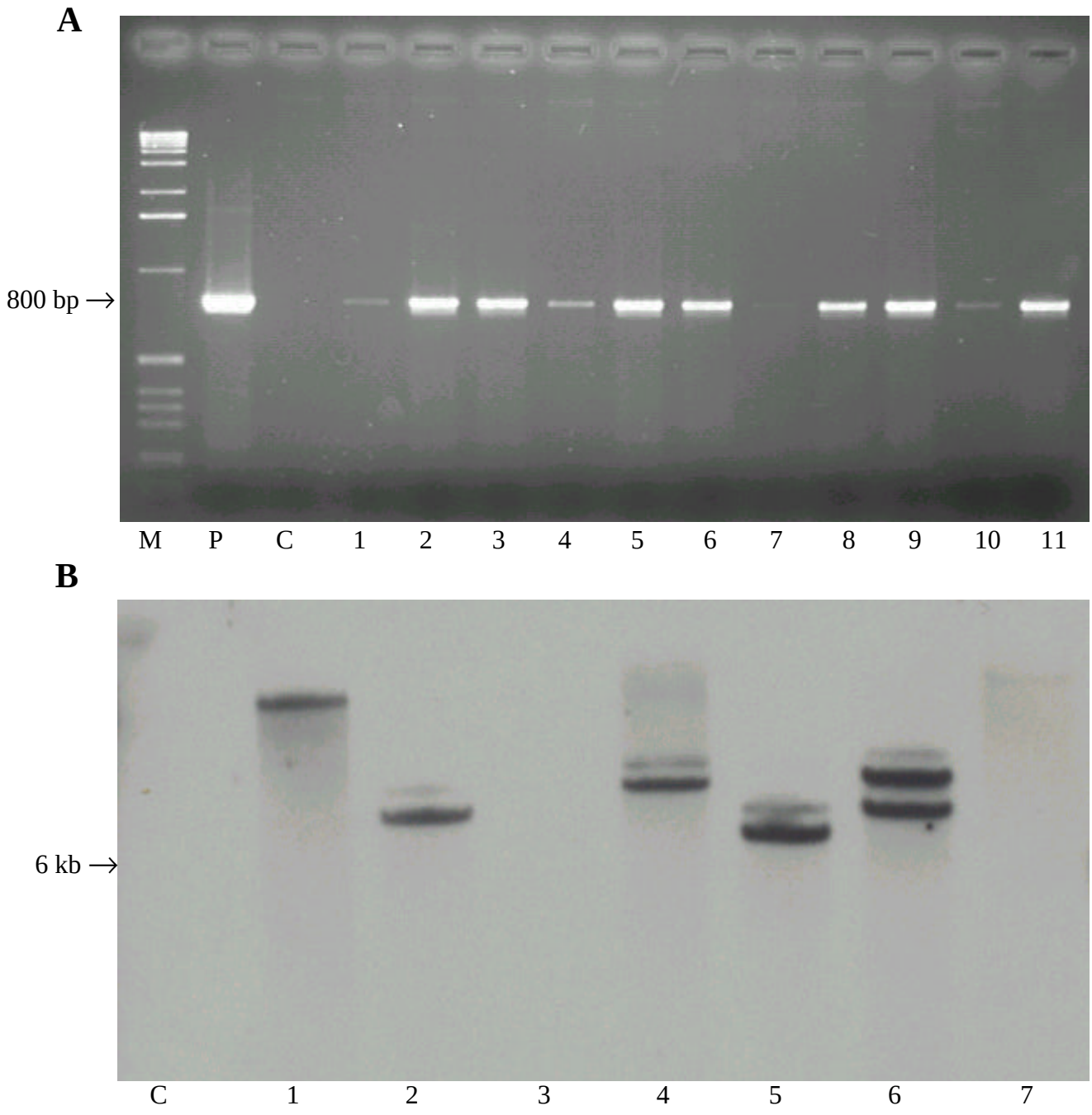


Figure 2- Analysis of the integration of GUS gene in the regenerated transgenic plants. **A-** Agarose gel of PCR-amplified 800-bp GUS fragment. *Lanes: M* 1-kb DNA marker, *P* pGA482GG plasmid, *C* untransformed control, *1-11* representative transformants of the regenerated transgenic plants. **B-** Southern blot analysis of the transgenic plants. Ten micrograms of the plant genomic DNA was digested with *Hpa*I and the filter was hybridized with DIG-labeled PCR-generated GUS fragment. *Lanes: C* untransformed control, *1-7* individual transformants.

Incorporation of carotenoid biosynthetic genes under constitutive expression

For introduction of the carotenoid biosynthetic genes into 'Duncan' grapefruit, the *C. paradisi* derived cDNA sequences of the phytoene synthase (CitPSY), phytoene desaturase (CitPDS), or lycopene-β-cyclase (CitLCY-B) genes were cloned into pCAMBIA 2301 under constitutive control either of the CaMV 35S or FMV 34S promoters, and used in *Agrobacterium*-mediated transformation experiments employing the optimized protocol. GUS assays were used to identify putative transformants; 60, 75, and 99 plants expressing GUS activity were obtained using the CitPSY, CitPDS, and CitLCY-B constructs, respectively (Table 2). PCR analysis using a pair of primers complementary to the *nptII* gene, located on the left T-DNA border, indicated that 75, 83, and 57% of the tested plants transformed the three constructs contained the entire T-DNA. The observable morphology of most of the transgenic plants was an increased pigmentation in the leaves compared to the wild type, although in some cases no differences were visualized.

Table 2- Genetic transformation of 'Duncan' grapefruit with the carotenoid biosynthetic genes phytoene synthase (CitPSY), phytoene desaturase (CitPDS), or lycopene-β-cyclase (CitLCY-B)

Construct	Number of segments cultured	Number of GUS^+ shoots	Shoots analyzed by PCR[a]	PCR^+ shoots
pCAMBIA 2301/CitPSY	2258	60	8	6
pCAMBIA 2301/CitPDS	2372	75	12	10
pCAMBIA 2301/CitLCY-B	2869	99	14	8

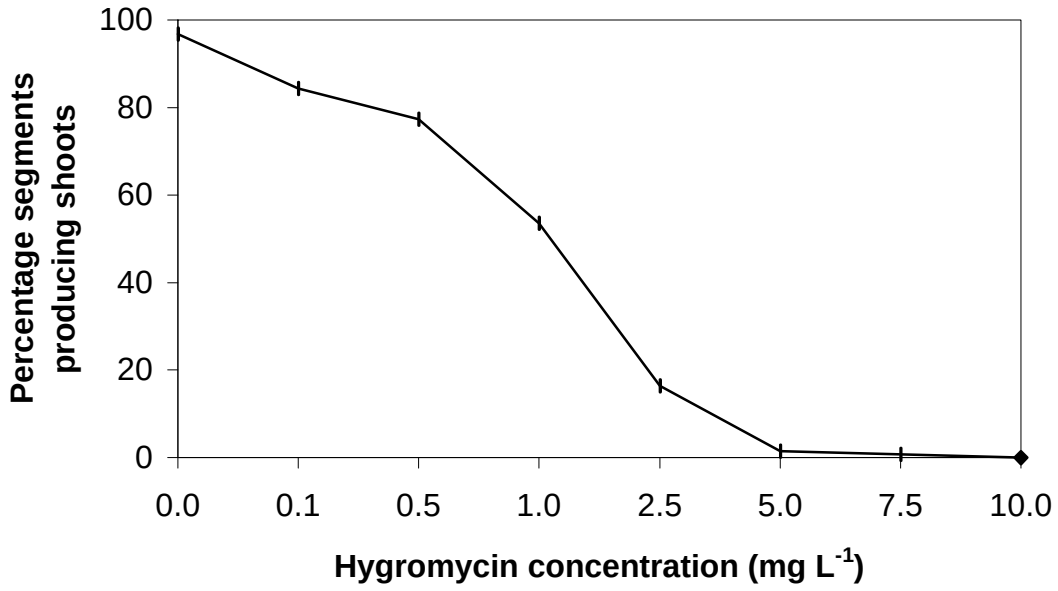
[a]Using *nptII* gene.

Hygromycin as a selective agent in citrus

The ineffectiveness of the antibiotic kanamycin as a selective agent has been reported as one of the main problems of citrus transformation (Moore et al. 1992, Peña et al. 1995a, Peña et al. 1995 b, Gutiérrez-E. et al. 1997, Peña et al.

1997). Attempts to improve kanamycin selection of citrus include prolonged continuous exposure of the explants to kanamycin (Peña et al. 1995b), addition of a liquid medium overlay containing kanamycin to culture plates (Gutiérrez-E. et al. 1997), and use of the antibiotic geneticin as selective agent, which is also detoxified by *nptII* activity (Peña et al. 1997). However, most of these techniques were laborious, led to the overgrowth of bacteria, or did not prevent the presence of escaped shoots. We evaluated hygromycin as a selective agent in citrus transformation, aiming not only to identify a more efficient antibiotic for recovering transformed citrus shoots, but also to test another selective agent that could be used for re-transformation of citrus plants already containing the *nptII* selectable marker gene. First, epicotyl explants of 'Duncan' grapefruit were cultured on media containing several concentrations of the antibiotic hygromycin in order to identify the most suitable concentration for selection of transformed shoots. As shown in Figure 3a, concentrations of 5.0 mg L^{-1} hygromycin or higher completely inhibited shoot formation. For evaluation of hygromycin on transformation efficiency, several concentrations starting from 5.0 mg L^{-1} were compared side-by-side with a control of 75 mg L^{-1} kanamycin, using two plasmids, pCAMBIA 1301 and pCAMBIA 2301, that differ only in the selectable marker genes *hptII* and *nptII*, respectively. Kanamycin was a more effective selective agent than any hygromycin concentration tested; 41% of the control regenerated shoots were GUS positive, while the best transformation rate using hygromycin was obtained at a concentration of 10 mg L^{-1}, where 33.3% of the regenerated shoots were GUS-positive. Although kanamycin was more effective as a selective agent than hygromycin, the results obtained were valuable since they demonstrated that hygromycin could be used in the genetic transformation of citrus, providing an alternative or additional selectable marker.

A



B

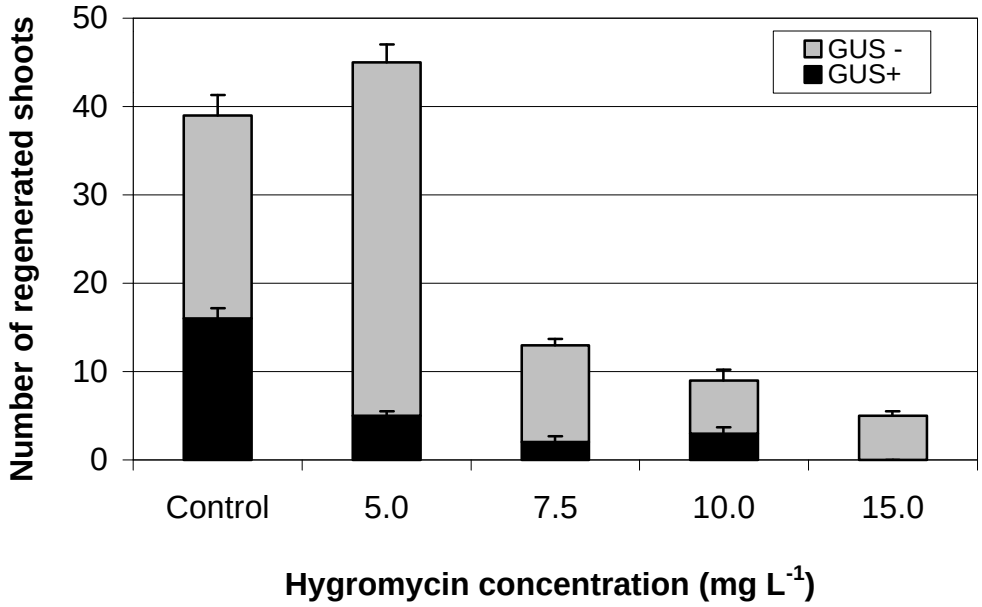


Figure 3- Use of the antibiotic hygromycin as selective agent. **A-** Effect of the antibiotic hygromycin on adventitious shoot regeneration from epicotyl explants of 'Duncan' grapefruit. **B-** Effect of the antibiotic hygromycin on efficiency of genetic transformation of 'Duncan' grapefruit. Explants were inoculated with EHA-105::pCAMBIA 1301 and then placed on selective regeneration medium containing from 5.0 to 15 mg L^{-1} hygromycin. *Control:* explants inoculated with EHA-105::pCAMBIA 2301 and then transferred to selective regeneration medium supplemented of 75 mg L^{-1} kanamycin. *Vertical bars* indicate the SE of two independent experiments.

BIBAC as a transformation vector in citrus

The BIBAC plasmid vector has been developed to permit the insertion of large fragments of DNA in plants (up to 150-kb) (Hamilton 1997), making it especially useful for the simultaneous introduction of several genes, such as those encoding the enzymes of metabolic pathways (Shibata and Liu 2000). The empty vector (BIBAC2) and the vector containing 150-kb of human DNA (BIBAC2.H150) were evaluated for citrus transformation. Few shoots were produced with either construct; the transformation rate was 0.3% with the empty vector and 0% with BIBAC2.H150 (Table 3). The low transformation efficiency with BIBAC2 empty vector may, in part, be due to the use of glucose rather than sucrose in the cocultivation and selective regeneration media; citrus cells may not use this source of carbon as efficiently as sucrose. However, this experiment indicated that this vector plasmid could be used in the metabolic engineering of citrus, although large numbers of explants might be required.

Table 3- Genetic transformation of 'Duncan' grapefruit with BiBAC2 empty vector and BiBAC2.H150 containing a 150-kb human genomic fragment

Plasmid[a]	Number of segments cultured[b]	Number (%) of segments producing shoots	Total number shoots produced	Number (%) of GUS^+ shoots	Number (%) of segments with GUS^+ shoots
BiBAC2	368	8 (2.2)	13	1 (7.7)	1 (0.3)
BiBAC2.H150	382	5 (1.3)	5	0	0

[a]Plasmids were in UIA-143 pMP90 pCH32 *Agrobacterium* construct.
[b]Data are summary of two independent experiments.

In summary, we have improved the transformation protocol for grapefruit, showed that genes from the carotenoid biosynthetic pathway were inserted into the citrus genome under constitutive expression, established that hygromycin can be used as a selective agent in our transformation system, and showed that the large insert BIBAC vector plasmid can be used to transform citrus, although without the addition of a large DNA construct. These experiments open the way for metabolic engineering of the carotenoid biosynthetic pathway in citrus, either

by insertion of multiple genes in the BIBAC plasmid or by multiple transformations using different selectable marker genes. Metabolic engineering of carotenoid biosynthesis has been achieved recently in tomato (Rosati et al. 2000, Römer et al. 2000, Fraser et al. 2001, Fraser et al. 2002), tobacco (Mann et al. 2000), canola (Shewmaker et al. 1999), and rice (Burkhardt et al. 1997, Ye et al. 2000). The growing interest in manipulating carotenoid biosynthesis in plants is mainly related to human nutrition, as precursors of vitamin A and natural antioxidants. In citrus, metabolic engineering of carotenoid biosynthesis has additional agronomic importance because it creates new possibilities for manipulating fruit and juice color and, possibly, plant growth. The constitutive expression of the phytoene synthase gene in tomato led to a reduction of plant height, likely caused by a competition for the same precursor between the gibberellin and carotenoid biosynthetic pathways (Fray et al. 1995).

Acknowledgments

We are grateful to Dr Jerry Slightom for providing the vector pGA482GG, Dr Elizabeth Hood for providing the *Agrobacterium* strains EHA-101 and EHA-105, Dr Carol Hamilton for providing the *Agrobacterium* constructs COR314 (UIA 143 pMP90 pCH32 BiBAC2) and COR356 (UIA 143 pMP90 pCH32 BiBAC2.H150), and Dr Vicente Febres for providing the vectors pCAMBIA 1301 and pCAMBIA 2301. We also appreciate the technical assistance provided by Ms Kim Ruesch. This research was supported in part by CAPES Foundation (Coordination of Higher Education and Graduate Training), Brasilia/Brazil, a USDA Special Grant to GAM, a USDA/UF Cooperative Agreement on Exotic Citrus Diseases to GAM, a FDACS/FCPRAC grant to GAM, and the Institute of Food and Agricultural Sciences at the University of Florida, USA. We gratefully acknowledge the ‘Sandwich’ Ph.D. scholarship to M.G.C. Costa by the CAPES Foundation, Brasilia/Brazil (process # BEX0600/00-8).

REFERENCES

An G (1987) Binary Ti vectors for plant transformation and promotor analysis. Methods Enzymol 153:292-305

Bond JE, Roose ML (1998) *Agrobacterium*-mediated transformation of the commercially important citrus cultivar Washington navel orange. Plant Cell Rep 18:229-234

Burkhardt PK, Beyer P, Wunn J, Kloti A, Armstrong GA, Schledz M, von Lintig J, Potrykus I (1997) Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis. Plant J 11:1071-1078

Cervera M, Pina JA, Juarez J, Navarro L, Peña L (1998) *Agrobacterium*-mediated transformation of citrange: factors affecting transformation and regeneration. Plant Cell Rep 18:271-278

Cervera M, Ortega C, Navarro A, Navarro L, Peña L (2000) Generation of transgenic citrus plants with the tolerance-to-salinity gene *HAL2* from yeast. J Hort Sci Biotechnol 75:26-30

Chomczynski P, Mackey K, Drews R, Wilfinger W (1997) DNAzol®: a reagent for the rapid isolation of genomic DNA. BioTechniques 22:550-553

Domínguez A, Guerri J, Cambra M, Navarro L, Moreno P, Peña L (2000) Efficient production of transgenic citrus plants expressing the coat protein gene of citrus tristeza virus. Plant Cell Rep 19:427-433

Farrand SK, Morchoe SP, McCutchan J (1989) Construction of an *Agrobacterium tumefaciens* C58 *recA* mutant. J Bacteriol 171:5314-5321

Fraser PD, Römer S, Kiano JW, Shipton CA, Mills PB, Drake R, Schuch W, Bramley PM (2001) Elevation of carotenoids in tomato by genetic manipulation. J Sci Food Agric 81:822-827

Fraser PD, Römer S, Shipton CA, Mills PB, Kiano JW, Misawa N, Drake RG, Schuch W, Bramley PM (2002) Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner. P Natl Acad Sci USA 99:1092-1097 DOI 10.1073/pnas.241374598

Fray RG, Wallace A, Fraser PD, Valero D, Hedden P, Bramley PM, Grierson D (1995) Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. Plant J 8:693-701

Ghorbel R, La-Malfa S, López MM, Petit A, Navarro L, Peña L (2001) Additional copies of *virG* from pTiBo542 provide a super-transformation ability to *Agrobacterium tumefaciens* in citrus. Physiol Mol Plant Pathol 58:103-110 DOI 10.1006/pmpp.2000.0318

Gutiérrez-E MA, Luth D, Moore GA (1997) Factors affecting *Agrobacterium*-mediated transformation in *Citrus* and production of sour orange (*Citrus aurantium* L.) plants expressing the coat protein gene of citrus tristeza virus. Plant Cell Rep 16:745-753

Hamilton C (1997) A binary-BAC system for plant transformation with high-molecular-weight DNA. Gene 200:107-116

Hood EE, Helmer GL, Fraley RT, Chilton MD (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J Bacteriol 168:1291-1301

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res 2:208-218

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5:387-405

Kaneyoshi J, Kobayashi S, Nakamura Y, Shigemoto N, Doi Y (1994) A simple and efficient gene transfer system of trifoliate orange (*Poncirus trifoliata* Raf). Plant Cell Rep 13:541-545

Luth D, Moore GA (1999) Transgenic grapefruit plants obtained by *Agrobacterium tumefaciens*-mediated transformation. Plant Cell Tiss Organ Cult 57:219-222

Mann V, Harker M, Pecker I, Hirschberg J (2000) Metabolic engineering of astaxanthin production in tobacco flowers. Nature Biotechnol 18:888-892

Moore GA, Jacono CC, Neidigh JL, Lawrence SD, Cline K (1992) *Agrobacterium*-mediated transformation of *Citrus* stem segments and regeneration of transgenic plants. Plant Cell Rep 11:238-242

Peña L, Cervera M, Navarro A, Pina JA, Durán-Vila N, Navarro L (1995a) *Agrobacterium*-mediated transformation of sweet orange and regeneration of transgenic plants. Plant Cell Rep 14:616-619

Peña L, Cervera M, Juarez J, Ortega C, Pina JA, Durán-Vila N, Navarro L (1995b) High efficiency *Agrobacterium*-mediated transformation and regeneration of *Citrus*. Plant Sci 104:183-191

Peña L, Cervera M, Juarez J, Navarro A, Pina JA, Navarro L (1997) Genetic transformation of lime (*Citrus aurantifolia* Swing): factors affecting transformation and regeneration. Plant Cell Rep 16:731-737

Peña L, Martin-Trillo M, Juarez J, Pina JA, Navarro L, Martínez-Zapater M (2001) Constitutive expression of *Arabidopsis LEAFY* or *APETALA1* genes in citrus reduces their generation time. Nature Biotechnol 19:263-267

Pérez-Molphe-Balch E, Ochoa-Alejo N (1998) Regeneration of transgenic plants of Mexican lime from *Agrobacterium rhizogenes* transformed tissues. Plant Cell Rep 17:591-596

Römer S, Fraser PD, Kiano JW, Shipton CA, Misawa N, Schuch W, Bramley PM (2000) Elevation of the provitamin A content of transgenic tomato plants. Nature Biotechnol 18:666-669

Rosati C, Aquilani R, Dharmapuri S, Pallara P, Marusic C, Tavazza R, Bouvier F, Camara B, Giuliano G (2000) Metabolic engineering of beta-carotene and lycopene content in tomato fruit. Plant J 24:413-419

Shewmaker CK, Sheehy JA, Daley M, Colburn S, Ke DY (1999) Seed specific overexpression of phytoene synthase: increase in carotenoids in other metabolic effects. Plant J 20:401-412

Shibata D, Liu Y-G (2000) *Agrobacterium*-mediated plant transformation with large DNA fragments. Trends Plant Sci 5:354-357

Slightom JL (1991) Custom polymerase –chain-reaction engineering of a plant gene expression vector. Gene 100:251-255

Spencer PA, Towers GHN (1991) Restricted occurrence of acetophenone signal compounds. Phytochemistry 30:2933-2937

Stachel SE, Messens E, Van Montagu M, Zambryski P (1985) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature 318:624-629

Sunilkumar G, Vijayachandra K, Veluthambi K (1999) Preincubation of cut tobacco leaf explants promotes *Agrobacterium*-mediated transformation by increasing *vir* gene induction. Plant Sci 141:51-58

Yang ZN, Ingelbrecht IL, Louzada E, Skaria M, Mirkov TE (2000) *Agrobacterium*-mediated transformation of the commercially important grapefruit cultivar Rio Red (*Citrus paradisi* Macf.). Plant Cell Rep 19:1203-1211

Ye X, Al-Babili S, Klöti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287:303-305

CONCLUSÕES GERAIS

Os resultados obtidos no presente estudo permitiram extrair as seguintes conclusões:

- As seqüências de cDNAs dos genes da rota biossintética de carotenóides isolados de *Citrus paradisi* apresentaram elevada homologia aos genes correspondentes em tomate, com identidade de 72-83% em nível de aminoácidos;
- O elevado conteúdo de espécies de hidrocarbonos insaturados de carotenóides encontrado em frutos de *C. paradisi* pode ser causada pela presença de transcritos aberrantes identificados em múltiplos locos, que decrescem o fluxo da rota em direção a formação de xantofilas;
- A expressão dos genes da sintase do fitoeno, desaturase do fitoeno e desaturase do ζ-caroteno variou entre tecidos (albedo e endocarpo) e cultivares (‘Duncan’ e ‘Flame’) durante o desenvolvimento do fruto, enquanto que o gene da ciclase-β do licopeno foi constitutivamente expresso em ambos os tecidos e cultivares;
- O padrão de acumulação de licopeno durante o desenvolvimento do fruto em variedades de *C. paradisi* que acumulam esse hidrocarbono

pode ser precisamente correlacionado com os níveis de expressão do gene da sintase do fitoeno no endocarpo;

- A diferenciação da coloração do fruto entre as cultivares de *C. paradisi* pode ser devida a mutações na seqüência aberta de leitura (inserções e deleções) e não a regulação transcricional diferencial, sendo a desaturase do fitoeno e/ou ciclase-ε do licopeno os genes candidatos pelas diferenças observadas;
- A morfogênese *in vitro* em segmentos de epicótilos de limão 'Cravo', pomelo 'Foster' e laranja 'Pêra' foi estimulada por BAP em concentrações ótimas de 0,5 a 1,0 mg l^{-1};
- A via de regeneração na extremidade de corte dos explantes foi dependente da concentração de BAP; explantes cultivados em meio contendo até 1,0 mg l^{-1} de BAP produziram brotos via organogênese direta, ao passo que explantes cultivados em meio contendo concentrações de BAP acima de 2,0 mg l^{-1} produziram brotos via organogênese indireta;
- O padrão geral de gradiente morfogênico observado ao longo do eixo do epicótilo foi uma maior resposta organogênica quando a distância dos explantes em relação ao nó cotiledonar aumentou;
- O padrão de gradiente morfogênico ao longo do eixo do epicótilo pode ser alterado em função do tipo e combinação de reguladores de crescimento utilizados no meio de cultura;
- A pré-cultura dos segmentos de epicótilos de 'Duncan' prévia a incubação dos explantes em *Agrobacterium* reduziu drasticamente a eficiência de transformação;
- O período de incubação dos explantes de 'Duncan' durante 20 minutos em solução de *Agrobacterium* aumentou sensivelmente o número total de brotos GUS^{+};
- A adição de 2,4-D ao meio de co-cultivo foi responsável pela maior freqüência de transformação obtida dentre os fatores avaliados;

- O período de co-cultivo superior a 2 dias diminuiu o número total de brotos GUS^+ obtidos;
- A concentração ótima de BAP no meio de regeneração seletivo foi de 2,0 mg l^{-1};
- O protocolo otimizado permitiu a obtenção de um elevado número de plantas transgênicas contendo os genes da sintase do fitoeno, desaturase do fitoeno ou ciclase-β do licopeno sob expressão constitutiva;
- Embora canamicina foi mais efetiva do que higromicina como agente seletivo, higromicina pode ser utilizada como um agente de seleção alternativo ou adicional;
- BiBAC pode ser utilizado como vetor de transformação em experimentos de engenharia metabólica em citros, embora o emprego de um grande número de explantes seja necessário.