CLEITON LOURENÇO DE OLIVEIRA

GENE EXPRESSION AND BIOAVALIABILITY OF CAROTENOIDS IN LETTUCE (*Lactuca sativa*)

LAVRAS – MG
2015

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitotecnia, área de concentração em Produção Vegetal, para obtenção do título de Doutor.

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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Oliveira, Cleiton Lourenço de.

Gene expression and bioavailability of carotenoids in lettuce (*Lactuca sativa*) / Cleiton Lourenço de Oliveira. – Lavras : UFLA, 2015.

112 p. : il.

Tese(doutorado)–Universidade Federal de Lavras, 2015.
Orientador(a): Luiz Antônio Augusto Gomes.
Bibliografia.

1. Metabolic pathway. 2. Lutein. 3. Beta carotene. 4. In vitro digestion. 5. Caco-2 cells. I. Universidade Federal de Lavras. II. Título.

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Aprovada em 27 de fevereiro de 2015.

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AO MEU DEUS CRIADOR E À MINHA NOSSA SENHORA DA CONCEIÇÃO APARECIDA, POR TODOS OS OBSTÁCULOS VENCIDOS E AOS ENSINAMENTOS ADQUIRIDOS

OFEREÇO

À minha esposa Franciene, por todo o amor, companheirismo e paciência.

Aos meus pais Dircésio e Laura e ao meu irmão Cléber, pelas orações e apoio.

À minha avó Georgina, pela fé que sempre em mim depositou.

DEDICO

AGRADECIMENTOS

Durante o nosso caminhar muitas opções nos são dadas e as escolhas podem não ser tão fáceis. Trocar a estabilidade do lar onde nasci pelo desafio de tentar a vida em outra cidade não foi fácil. As lágrimas derramadas por aqueles que de certa forma queriam o menino recém-crescido por perto, mas ao mesmo tempo queriam o melhor para ele, me fizeram pensar muito frente a cada desafio inicial. Mas certamente, hoje posso ver que tudo valeu muito a pena e as coisas fluíram muito bem desde o início. Nesse sentido, inicio esses agradecimentos deixando minha gratidão a Deus, que através da intercessão de Nossa Senhora da Conceição Aparecida sempre me mostrou o melhor caminho e me concedeu tudo toda vez que pedi. Agradeço aos meus pais Dircésio e Laura por todo o apoio e por tudo que fizeram, mesmo diante das dificuldades para que eu pudesse estudar. Agradeço também ao meu irmão Cléber pelo apoio e por auxiliar tantas vezes nas tarefas da roça quando precisei sair de lá.

Agradeço a todos os meus tios e tias, primos e amigos de Areado – MG, principalmente às tias Ritinha e Lucilene pelas orações e apoio, ao primo Tiago pelo grande incentivo incial, e ao primo Fábio pelas risadas e descontrações que aliviaram as eventuais tensões. Agradeço à vó Georgina por sempre, sempre me apoioar e depositar muita confiança em mim. Certamente todo esse apoio fez um grande diferencial.

Agradeço a minha esposa, Franciene que esteve ao meu lado me apoiando, aconselhando e amparando diante das dificuldades, sempre com muita paciência.

Aos amigos de Lavras, aos meus sogros Márcio e Consuelo e minha cunhada Jéssica, por todo o apoio e auxílio.

Às escolas Municipal João Luiz Alves, Estadual João Lourenço e Colégio Nossa Senhora das Graças em Areado por terem feito parte da minha formação inicial.

À Universidade Federal de Lavras, pela oportunidade dos cursos de graduação, mestrado e doutorado. À Coordenação de Pessoal de Nível Superior pela concessão das bolsas de estudos.

À Hortiagro Sementes S/A, principalmente ao Paulo Moretto e ao Vicente pelo auxílio técnico na execução dos ensaios preliminares.

Ao meu orientador Prof. Dr. Luiz Antônio Augusto Gomes por depositar tanta confiança na execução dos trabalhos, além da excelente orientação, calma, sabedoria, paciência, muitos conselhos e amizade.

Ao Prof. Dr. Matheus de Souza Gomes, da Universidade Federal de Uberlância por todo o auxílio nas análises de bioinformática e pelo contato estabelecido para a realização do doutorado sanduíche.

Ao prof. Dr. Antônio Chalfun Júnior pela disposição e espaço cedido no laboratório para o treinamento inicial com biologia molecular.

À banca examinadora pela disposição e contribuições com o trabalho.

Aos amigos e colegas do grupo de melhoramento genético de alface e feijão-vagem André, Daniela, Daniele, Deborah, Flávia, Gabriel, Giuliana, Hugo, Luciana, Marcelo, Márcia, Mariana, Marina, Natália, Pedro, Renê, Raisla, Roberta e Rodolfo, por toda a amizade e trabalho em equipe.

Meu sincero Muito Origado a todos vocês!

ACKNOWLEDGEMENTS

I'd like to say thank you for everyone in the Genetics & Biotechnology Lab and Plant & AgriBiosciences Research Centre (PABC) at the National University Ireland Galway. My co-supervisor Dr. Charles Spillane for all the orientation and Dr. Ronan Sulpice for all advices and tips. To all my friends and colleagues that I knew there. Antoine Fort, Attia Fatima, Bruno Henrique, Dawn Howard, Edna Curley, Ester Fonseca, Galina Brychkova, Girum Azmach, Julianna Xavier, Marijke Hummel, Martin Braud, Merci Kitavi, Peter McKeown, Peter Ryder, Reetu Tuteja, Sandesh Rao, Sandra Raimundo, Simrat Kaur, Xiaozhen Han and Zewdy Gebremedhin.

Specially, thanks to Alberto Abrantes, Anish Kumar and Marc Deletre for the true friendship and good times. Was really great work with you guys!

Thank you very much for all!

“Não há problema que não possa ser solucionado pela paciência”

Chico Xavier

RESUMO GERAL

Foram avaliados níveis de transcrição de dezessete genes envolvidos na via de biossíntese de carotenoides durante o desenvolvimento de folhas novas e velhas de seis genótipos de alface contrastantes para intensidade de verde nas folhas. Diferenças significativas na concentração de carotenoides foram identificadas entre cultivares e entre folhas velhas e novas. O acúmulo de β-caroteno aumentou ao longo do desenvolvimento das plantas entre o tamanho de muda até 40 dias após a semeadura e diminuiu quando o tamanho comercial foi atingido. Contrariamente, os níveis de luteína não variaram ao longo do desenvolvimento. As análises de correlação identificaram genes da via, os quais os níveis de expressão poderiam ser utilizados como marcadores para a predição das concentrações finais de clorofila, luteína e β-caroteno no tamanho comercial de plantas de alface. Nesse sentido, *GGPPS*, *PSY1* e *LCY-β1* foram significativos na predição da concentração de carotenoides em plantas comerciais de alface, enquanto os níveis de expressão de *CRTISO* e *LCY-β2* no tamanho comercial foram correlacionados com o acúmulo de carotenoides nessa fase. Esses resultados sugerem genes candidatos a marcadores para seleção de plantas de alface com altos níveis de clorofila total, luteína e β-caroteno e fornecem ainda uma projeção para estudos de atividade funcional da síntese e elevação dos teores de carotenoides em vegetais folhosos. Na segunda parte, foram avaliadas a bioacessibilidade e a biodisponibilidade de luteína e β-caroteno presentes nas folhas novas e velhas dos mesmos genótipos utilizados na primeira parte em tamanho comercial, utilizando um modelo de digestão *in vitro*, seguido do isolamento da fração micelar e quantificação das porcentagens de carotenoides absorvidos por células de adenocarcinoma intestinal humano. Os resultados indicaram que diferenças na concentração inicial de carotenoides não são proporcionais à absorção e transporte pelo intestino humano. A destruição da matriz alimentar dos genótipos de alface por meio de cocção resultou em perdas na concentração de carotenoides transferidos para a fase líquida da digestão, mas essas perdas foram compensadas por um aumento de oito vezes na absorção dos carotenoides pelas células comparando com os resultados de folhas frescas digeridas sem tratamento térmico. A menos que a matrix alimentar seja rompida, a absorção de carotenoides presente em alface biofortificado ocorre em taxas muito baixas, similares às de genótipos com baixa concentração de carotenoides. Esses resultados sugerem que o melhoramento genético para biofortificação deve estar relacionado com esforços para aumentar a biodisponibilidade dos carotenoides acumulados na matriz alimentar.

Palavras-chave: Alface. Carotenoides. Via Metabólica. Luteína. β-caroteno. Digestão *in vitro*. Biodisponibilidade. Células caco-2.

GENERAL ABSTRACT

We evaluated transcript levels of seventeen genes involved in the biosynthesis of carotenoids during the development of inner and outer leaves of six contrasting lettuce genotypes for green color intensity. Significant differences in the concentration of carotenoids were identified among leaves age and cultivars. The accumulation of β-carotene increased over the development of the plants between seedling size until 40 days after sowing and decreased when the commercial size was reached. In contrast, lutein levels did not vary throughout the development. Correlation analyzes identified pathway genes which expression levels could be used as biomarkers for the prediction of the final concentrations of chlorophyll, lutein and β-carotene in the commercial size lettuce plants. In this regard, *GGPPS*, *PSY1* and *LCY-β1* were significant in predicting the concentration of carotenoids in commercial lettuce plants, while *CRTISO* and *LCY-β2* expression levels in commercial size were correlated with the accumulation of carotenoid in this stage. These results suggest the candidate genes that could be used as biomarkers for selection of lettuce plants with high levels of chlorophyll, lutein and β-carotene and provide a projection for functional studies of synthesis and accumulation of carotenoids in green leafy vegetables. In the second part, we evaluated the bioaccessibility and the bioavailability of lutein and β-carotene stored at commercial size in inner and outer leaves of the same genotypes used in the first experiment, using an *in vitro* digestion model followed by the isolation of micellar fraction and quantification of carotenoids percentages absorbed by the human intestinal adenocarcinoma cells. The results indicated that differences in the initial concentration of carotenoids are not related to absorption and transport by the human intestine. The disruption of the lettuce genotypes food matrix through cooking procedure resulted in losses of carotenoids transferred to the liquid phase of digestion, but these losses were compensated for by an eight-fold increase in the absorption of carotenoids from the cells comparing with the results of fresh leaves digested without heat treatment. Unless the food matrix is broken, the absorption of carotenoids in biofortified lettuce occurs in a very low rate, similar to low carotenoids genotypes. These results suggest that breeding programs for biofortification should be related to efforts to increase the bioavailability of carotenoids present in the food matrix.

Keywords: Lettuce. Carotenoids. Metabolic pathway. Lutein. β-carotene. *In vitro* digestion. Bioavailability. Caco-2 cells.

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1 INTRODUÇÃO

A alface (*Lactuca sativa* L.) é a espécie folhosa mais consumida mundialmente, apresentando grande importância econômica e, mais recentemente, importância funcional no organismo. Sabe-se hoje que vegetais folhosos são importantes fontes de vitaminas e minerais, tais como vitamina A e C e potássio, que podem estar envolvidos em atividades pró-vitamínicas e antioxidantes, além de serem fontes de fibras insolúveis.

Nos últimos anos, a alface tem sido alvo de estudos quantitativos e qualitativos que a consideram como importante fonte de carotenoides, que estão entre os fitoquímicos que mais proporcionam benefícios à saúde humana, principalmente luteína e β-caroteno, que estão associados à prevenção de diversas enfermidades, principalmente relacionadas a problemas de visão. A luteína é um carotenoide do grupos das xantofilas que é diretamente armazenada na mácula do olho e sua deficiência pode levar à redução da acuidade visual. Já o β-caroteno é o principal carotenoide do grupo dos carotenos que apresentam atividade pró-vitamina A e sua deficiência está relacionada à redução da capacidade antioxidante do organismo, infecções pulmonares e, em casos mais severos, cegueira noturna.

Na literatura existem ainda poucos trabalhos que tratam da identificação de genes envolvidos na via metabólica de síntese e degradação de carotenoides em alface. A identificação de genes que são expressos ao longo do ciclo da planta pode ser uma importante ferramenta para identificação indireta de genótipos com altos teores de carotenoides. Juntamente a isso, apesar de uma grande variação já ter sido identificada para teores de luteína e β-caroteno em alface (CASSETARI et al., 2013; MOU, 2005), bem como uma correlação positiva com a intensidade de coloração verde, quase nada se sabe a cerca da capacidade desses carotenoides serem, de fato, absorvidos pelo organismo humano. Essa capacidade de absorção está condicionada ao rompimento da

matriz alimentar, em que os carotenoides são armazenados nas plantas, além dos fatores quantidade de carotenoides, lipídeos, tipo de processamento/cocção, etc., o que sugere diferença na absorção entre carotenoides advindos de frutos e vegetais folhosos (VAN HET HOF et al., 2000). Em vegetais folhosos, o armazenamento dos carotenoides ocorre nos cloroplastos, onde estão associados à captação de energia luminosa e proteção de clorofilas. Já em órgãos vegetais não fotossintetizantes classificados como fontes seguras de carotenoides, ao contrário de órgãos vegetais fotossintetizantes, os carotenoides são armazenados nos cromoplastos e estão associados a funções de reserva.

Objetivou-se no presente trabalho identificar possíveis genes relacionados à síntese e degradação de carotenoides em alface, bem como a quantificação e avaliação da biodisponibilidade *in vitro* desses carotenoides. A identificação de genes relacionados à síntese, acumulação e degradação de carotenoides em plantas de alface pode ser utilizada como uma ferramenta para a seleção precoce de genótipos promissores, além de resultar em economia de recursos que são necessários para a condução das plantas até o tamanho comercial para a avaliação convencional dos teores de carotenoides. Já o estudo da biodisponibilidade é importante para saber a real quantidade dos carotenoides presentes em plantas de genótipos contrastantes de alface que está, de fato, disponível para utilização no metabolismo do corpo humano. Assim, foram realizados dois trabalhos com seis genótipos de alface com diferenças visíveis na intensidade da coloração verde. No primeiro, avaliaram-se os níveis de luteína, β-caroteno e clorofila total em três estágios do desenvolvimento da planta, sendo tamanho de muda, 40 dias após a semeadura e tamanho comercial, com amostragem em folhas novas e velhas das plantas nos dois últimos estágios. Esses teores foram correlacionados com a expressão de genes da via metabólica de carotenoides, previamente selecionados por análise de busca de etiquetas de sequências transcritas (EST) de genes de via de síntese de carotenoides em

crisântemo (*Crysanthemum morifolium*), maçã (*Malus domestica*) e Arabidopsis (*Arabdopsis thaliana*).

No segundo trabalho aplicou-se um modelo de digestão *in vitro* em folhas frescas e cozidas dos mesmos genótipos de alface para avaliar o efeito de matriz alimentar dos genótipos na capacidade de liberar os carotenoides armazenados nas folhas após a digestão (bioassessibilidade), bem como a aborção de luteína e β-caroteno através de uma simulação de absorção intestinal utilizando-se o modelo de monocamada de células epiteliais diferenciadas de adenocarcinoma intestinal humano da linhagem Caco-2 (biodisponibilidade).

2 REFERENCIAL TEÓRICO

2.1 Produção de alface no Brasil e no mundo

A alface é considerada o vegetal com maior destaque dentro do grupo dos vetegais folhosos, apresentando o maior consumo e importância econômica no mundo, principalmente para países da Ásia, América Central, América do Norte e Europa (LEBEDA et al., 2007). A produção em 2011 foi da ordem de 23.2 milhões de toneladas anuais, liderando no *ranking* mundial a China com 13.4 milhões de toneladas, seguida pelos Estados Unidos com 4.07 milhões e Índia com 1.06 milhões (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS - FAO, 2013).

No Brasil, segundo dados do Anuário da Agricultura Brasileira (2014), no ano de 2012 foram comercializadas 41.925 toneladas de alface no CEAGESP (ANUÁRIO..., 2014) em uma área total estimada em de 39.000 ha (INSTITUTO DE ECONOMIA AGRÍCOLA DO ESTADO DE SÃO PAULO, 2014), sendo os maiores produtores os estados de São Paulo, Minas Gerais e Rio de Janeiro com 31%, 27% e 7% da produção nacional, respectivamete. Os dados mais recentes disponíveis mostram que a preferência ainda é pela alface do tipo crespa, que respondeu por 48% do total comercializado no ano de 2010 (ANUÁRIO..., 2012). A importância da alface conhecida como - tipo americana - no Brasil nos útimos anos, bem como o crescimento na demanda por esse tipo, é atribuída à introdução desse tipo de alface no Brasil via cultivares norte-americanas na transição da década de 1960 para 1970 (SALA; COSTA, 2012).

2.2 Histórico e melhoramento genético de alface

O melhoramento genético de alface teve início já no seu berço de origem, localizado no oriente médio onde hoje estão Egito e Irã, com seleções

feitas por humanos habitantes da região. Essas primeiras seleções resultaram num grande *gene pool* de *Lactuca serriola*, seguidas por introgressões de genes de outras espécies do gênero *Lactuca* (LINDQVIST, 1960).

Mais tarde, a alface foi distribuída no império romano através do mar Mediterrâneo, vindo a ser introduzida no ocidente do continente europeu, provavelmente no início do século XV, sendo em seguida, trazida para a América por Cristóvão Colombo em 1494 (RYDER, 2002). No Brasil a introdução da alface foi feita pelos portugueses em 1650 (SALA, 2011).

A primeira cultivar de alface lançada no Brasil foi a Brasil 48, do Instituto Agronômico de Campinas (IAC), na década de 1970, com resistência ao *Lettuce mosaic virus* (LMV) e ao calor (NAGAI, 1979). Até a década de 1980 o padrão de consumo de alface no país era o tipo lisa, sendo as principais cultivares a White Boston e a San Rivale, ambas centenárias (SALA, 2011). No entanto, o melhoramento genético já possuía certa expressão desde a década de 1960 com os trabalhos realizados no Instituto Agronômico de Campinas – IAC por Iroshi Nagai, com esforços voltados inicialmente ao desenvolvimento de cultivares resistentes a viroses. Na década de 1990, Nagai desenvolveu a série Brasil 500, com genótipos de folhas crespas, resistentes a viroses e com tolerância ao florescimento precoce (MELO; MELO, 2003). O desenvolvimento dessa série permitiu que o Brasil se tornasse mais independente das empresas internacionais na produção de sementes, além de os materias desenvolvidos terem sido utilizados como base de diversos programas de melhoramento (GOMES, 1999).

Até o final da década de 1990, o melhoramento genético de alface estava voltado basicamente para o desenvolvimento de cultivares com resistência a doenças, além de, principalmente, o desenvolvimento de cultivares com maior resistência ao pendoamento precoce que viabilizasse o cultivo de alface durante todo o ano, inclusive durante a primavera e o verão (FILGUEIRA, 2000). Ao longo dos anos foram realizados trabalhos que

identificaram genes relacionados ao controle dessas características, como a série *Ef* que contola o florescimento (RYDER, 1986), os genes da série *Dm*, relacionados ao controle do míldio (*Bremia lactucae*) (FARRARA; ILOT; MICHELMORE, 1987; LEBEDA; SCHWINN, 1994), os genes de resistência ao *Lettuce Mosaic Virus* (LMV) (STANGARLIN, 1994) e o genes de resistência a nematoides (GOMES; MALUF; CAMPOS, 2000). No entanto, ultimamente a pesquisa voltada para o melhoramento de alface tem sido globalizada e concentrada nas estações experimentais localizadas nos países-sede das multinacionais e não mais no Brasil, de modo que os ensaios não mais têm sido realizados nas condições brasileiras, nos sistemas de cultivos e para as raças peculiares de patógenos que ocorrem no Brasil (SALA; COSTA, 2012). Essas empresas adotam a estratégia de globalização da pesquisa e não têm tido sucesso com o desenvolvimento de cultivares adaptadas às nossas condições de cultivo.

Por outro lado, alguns exemplos de sucesso têm sido obtidos com os programas de melhoramento de alface desenvolvidos no Brasil por algumas empresas nacionais e algumas instituições de pesquisa, visando à obtenção e liberação de cultivares adaptadas às nossas condições de cultivo. O lançamento de cultivares nacionais, estimulado pelo Serviço Nacional de Proteção de Cultivares (SNPC) do Ministério da Agricultura brasileiro tem possibilitado a oferta de cultivares com tolerância ao pendoamento precoce, adaptação às condições climáticas de verão com elevada pluviosidade e resistência às principais doenças, o que vem permitindo o cultivo dessas cultivares pelos produtores e contribuindo para dar sustentabilidade ao cultivo de alface no Brasil (SALA; COSTA, 2012).

Na última década, trabalhos relacionados ao incremento de teores de carotenoides pró-vitamina A têm tomado certa importância. A presença de variabilidade entre cultivares para teores de luteína e β-caroteno, bem como uma correlação entre β-caroteno e clorofila foram demonstrados, sugerindo um

potencial uso do teor de clorofila como indicador indireto do teor de carotenoides, bem como a viabilidade do melhoramento genético em alface para aumento dos teores desses compostos (MOU, 2005).

Também no Brasil nos últimos anos, a preocupação com a deficiência de vitamina A, especialmente nas regiões menos desenvolvidas, têm levado ao desenvolvimento de cultivares com índices mais altos de carotenoides pró-vitamina A, o que resultou na obtenção da cultivar Uberlândia 10.000 que possui, em cada 100 gramas de folhas frescas, mais de 10.000 unidades internacionais (U.I.) de vitamina A, equivalentes a 36 mg de β-caroteno por 100 gramas de folhas frescas (SOUSA et al., 2007). As linhagens foram selecionadas baseando-se em caracteres morfológicos, como coloração das folhas, resistência ao pendoamento precoce, sabor doce e adaptação a variações no pH do solo. A cultivar Uberlândia 10.000 originou-se de uma série de seleções a partir do cruzamento de 'Maioba' e 'Salad Bowl-Mimosa', que deu origem a cultivar Moreninha-de-Uberlândia que, apesar de alto teor de vitamina A, possuía características inadequadas às exigências do consumidor, sendo então cruzada com a cultivar Vitória de Santo Antão, que resultou finalmente na cultivar Uberlândia 10.000 (SOUSA et al., 2007).

As tendências atuais e futuras são atrelar as técnicas clássicas às de biotecnologia, visando à otimização dos resultados obtidos no melhoramento genético de alface para as mais diversas características. Esse emprego pode contribuir significativamente para o conhecimento básico da cultura e do caráter estudado, além da geração e desenvolvimento de produtos melhorados (SOUSA et al., 2007).

2.3 Melhoramento genético para a biofortificação de alimentos

As pesquisas atuais relacionadas à biofortificação de alimentos estão ligadas ao desenvolvimento de cultivares de culturas básicas com altos níveis de

micronutrientes, utilizando-se de práticas de melhoramento genético convencionais juntamente as práticas modernas de biotecnologia (NESTEL et al., 2006).

O potencial para aumentar o teor de micronutrientes em culturas de importância básica na alimentação humana por meio do melhoramento genético convencional existe (GRAHAM et al., 1999; GRAHAM; WELCH; BOUIS, 2001). Variância genética para concentração de β-caroteno e outros carotenoides, bem como ferro, zinco e outros minerais existem entre cultivares, tornando possível a seleção de materiais de interesse (MOU, 2005; NESTEL et al., 2006). Além disso, a concentração de micronutrientes se mostra estável em ambientes diferentes, além de que o controle genético é simples, o que torna viável o melhoramento genético visando ao aumento simultâneo de mais de um micronutriente (NESTEL et al., 2006).

A biofortificação, além de proporcionar melhoria no aspecto da deficiência de determinado composto em populações menos favorecidas, apresenta ainda uma série de vantagens. Primeiramente, apenas com a mesma quantidade de alimentos consumidos regularmente é possível melhorar a quantidade de micronutrientes ingeridos. Um bom exemplo é a batata doce, em que, nos países dependentes dessa cultura, o consumo diário de 200 g de raízes cozidas não é suficiente para suprir os 250 µg de retinóis equivalentes (3 mg de β-caroteno) diários recomendados para crianças. A simples substituição da bata doce comum por batata doce da polpa alaranjada já seria mais que suficiente para suprir essa necessidade. No entanto, essas cultivares costumam apresentar baixo teor de matéria seca e consumidores africanos preferem batatas doces com altos teores (JOLLIFFE, 2004). Outro exemplo é o arroz “Golden Rice”. Enquanto β-caroteno não é encontrado em nenhuma variedade convencional de arroz, o “Golden Rice”, desenvolvido por meio de transgenia apresenta 37$\mu g.g^{-1}$ de carotenoides, dos quais 31 $\mu g.g^{-1}$ são β-caroteno (PAINE et al., 2005). Uma segunda vantagem é que, apesar de um determinado custo inicial, depois de algum tempo investido no

desenvolvimento de sementes capazes de passar o caráter incrementado para as gerações seguintes, os custos passam a serem baixos e o germoplasma pode então ser distribuído internacionalmente. Em terceiro, uma vez divulgada e estabelecida em determinada região, a cultura biofortificada se torna altamente sustentável. Variedades melhoradas nutricionalmente tendem a ser cultivadas e consumidas ano após ano, mesmo que a atenção de órgãos governamentais e fundos internacionais diminuam ao longo dos anos. Em quarto, a biofortificação fornece meios viáveis para atingir populações subnutridas em áreas rurais remotas, através da distribuição de alimentos biofortificados para pessoas com acesso limitado a estabelecimentos comerciais. Por fim, o melhoramento genético para biofortificação tem por meta também não resultar em perdas de rendimento no campo (GRAHAM; WELCH; BOUIS, 2001).

A biofortificação de alimentos ainda possui algumas vantagens indiretas, como o aumento da produtividade e melhoria da qualidade ambiental. Alguns minerais alvos da biofortificação, como zinco, por exemplo, são essenciais para o aumento da resistência das plantas contra algumas doenças e outros estresses ambientais, resultando em um melhor desenvolvimento inicial e, consequentemente, maiores produtividades (NESTEL et al., 2006). No entanto, apesar das inúmeras vantagens, o melhoramento genético para biofortificação ainda precisa vencer a barreira da aceitação em alguns casos específicos. Quando a concentração de determinado nutriente resulta em uma mudança visível de coloração, associada ao aumento do teor de provitamina A, por exemplo, ou ainda mudanças no teor de matéria seca, uma recusa por parte do agricultor e/ou do consumidor final pode ocorrer. Nesse caso, a adoção de cultivares biofortificadas que apresentem mudanças visíveis no fenótipo pode se dar em função da aceitação. Por outro lado, quando a biofortificação é feita no sentido da elevação de teores de ferro ou zinco, nenhuma mudança visual é percebida, o que torna a aceitação mais fácil (NESTEL et al., 2006).

O melhoramento genético visando à biofortificação requer que as pesquisas de campo estejam constantemente ligadas às pesquisas de nutrição e saúde humana (BOUIS, 2003). Isso sugere que o desenvolvimento de novas variedades para combinar o melhor de aspectos nutricionais e caracteres agronômicos para uma determinada cultura não é o suficiente. É necessário que o melhoramento esteja ainda em conjunto com estudos realizados na área de engenharia de alimentos e nutrição, para medir a retenção de nutrientes em alimentos crus e processados para a seleção de linhas que não só possuam altos teores de um determinado micronutriente ou vitamina, mas que também sejam promissoras na biodisponibilidade em humanos (NESTEL et al., 2006). A aplicação de novas técnicas de biotecnologia, genômica, genética e biologia molecular que possibilitem a identificação e o entendimento de genes envolvidos nas vias metabólicas de importância nutricional, incluindo aqueles que atuam como promotores e inibidores da absorção de nutrientes apresenta-se como uma opção viável. O melhoramento genético nesse sentido é a tendência futura, uma vez que somente o conhecimento da composição nutricional da planta não é suficiente para assegurar que os micronutrientes e vitaminas serão, de fato, absorvidos pelo organismo.

2.4 Análise da expressão gênica

Nos últimos anos, técnicas avançadas de biologia molecular vêm sendo aplicadas como ferramentas para a identificação de fatores envolvidos na expressão de determinado caráter nos mais diversos organismos. Em plantas, a aplicação da técnica de PCR em tempo real ganhou expressividade ultimamente, sendo utilizada na quantificação da expressão de genes que atuam em vias metabólicas dos mais diversos caracteres.

O desenvolvimento da técnica de PCR (reação em cadeia da polimerase) se deu pela clonagem *in vitro* proposta em 1984 por Kary Mullis (MULLIS; FALOONA, 1987), que é a polimerização e multiplicação

exponencial de regiões ou sequencias específicas de um material genético. Essa descoberta foi de grande importância no avanço de técnicas moleculares ao longo dos anos, dando origem em 1993 à reação de amplificação em tempo real (qRT-PCR), uma variante da reação de PCR convencional que representou grande avanço nos métodos moleculares, particularmente por facilitar sobremaneira as tarefas de quantificação da expressão gênica em determinado tecido ou amostra biológica (GIBSON; HEID; WILLIAMS, 1996; HEID et al., 1996; HIGUCHI et al., 1993). A qRT-PCR é considerada uma das técnicas mais sensíveis e precisas na detecção e quantificação de transcritos expressos em determinado momento celular (GACHON; MINGAM; CHARRIER, 2004). O processo é baseado na transcrição reversa seguida de reações sucessivas em cadeia, utlizando uma DNA-polimerase com a incorporação de moléculas fluorescentes de natureza covalente, ligadas às fitas duplas de DNA geradas no processo, as quais podem ser quantificadas durante a reação. É uma ferramenta abrangente e cada vez mais utilizada devido à sua alta sensibilidade, boa reprodutividade, amplo dinamismo e vasta gama de quantificação (BUSTIN, 2000; LOCKEY; OTTO; LONG, 1998).

O emprego da qRT-PCR tem crescido ultimamente em diversos estudos que visam à mensuração da expressão de genes alvos relacionados aos mais diversos fatores dos mais diversos organismos. Por exemplo, essa técnica tem sido empregada com sucesso na discriminação de perfis de expressão de genes relacionados à síntese e/ou degradação de carotenoides em várias culturas de importância econômica como tomate, cenoura, abobrinha, maçã, kiwi, etc., bem como em *A. thaliana* (ÁGUILA RUIZ-SOLAA; RODRÍGUEZ-CONCEPCIÓNA, 2012; AMPOMAH-DWAMENA et al., 2009, 2012; CAZZONELLI; YIN; POGSON, 2009; CLOTAULT et al., 2008; FAILLA; HUO; THAKKAR, 2008; GIULIANO; BARTLEY; SCOLNIK, 1993; KATO et al., 2006; KISHIMOTO; OHMIYA, 2006; OBRERO et al., 2013).

2.5 Digestão *in vitro* e utilização de células de linhagem Caco-2 na quantificação da biodisponibilidade de carotenoides

A utilização da digestão *in vitro* para simular a digestão gástrica e intestinal que ocorre naturalmente no organismo humano é uma ferramenta importante para simular o que ocorre com o alimento após a ingestão. Em se tratando de carotenoides presentes em vegetais, esses necessitam ser eficientemente dispersos no trato digestivo e solubilizados em micelas, o que é um pré-requisito para a absorção pelo epitélio intestinal (NAGAO, 2014). Sendo assim, a digestão *in vitro* é uma ferramenta que tem sido amplamente utilizada em diversos trabalhos para quantificar a bioacessibilidade, que é a quantidade de carotenoides que fica disponível para ser absorvida pelo epitélio intestinal humano (COURRAUD et al., 2013; HEDREN; DIAZ; SVANBERG, 2002; LEMMENS et al., 2009; RICH et al., 2003; RYAN et al., 2008).

Em vegetais, essa bioacessibilidade de carotenoides está relacionada à composição e arranjos dos carotenoides na matriz alimentar, em que os mesmos estão armazenados. Dessa forma, a digestão juntamente a maneira de preparo desses vegetais, além da composição dos carotenoides podem afetar a micelarização e, consequentemente, a absorção dos mesmos (VAN HET HOF et al., 2000). Por exemplo, as quantidades de β-caroteno e retinol presentes no soro sanguíneo podem aumentar mais em indivíduos alimentados com β-caroteno sintético, do que em indivíduos alimentados com vegetais folhosos contendo a mesma quantidade de β-caroteno (DE PEE et al., 1995).

Para quantificar a biodisponibilidade de carotenoides, geralmente são empregados ensaios *in vivo* que utilizam tanto humanos quanto outros animais para fornecerem informações acerca da biodisponibilidade, através da quantificação de nutrientes no soro sanguíneo (disponível para utilização), em tecidos animais (estocados) e, em caso de estudos com isótopos, na excreção. No entanto, esse tipo de estudo tem se mostrado caro e burocrático. Como

alternativa, a utilização de células epiteliais de carcocinoma intestinal humano da linhagem Caco-2 tem crescido muito nos últimos anos nos mais variados campos de pesquisa, principalmente na área de ciências farmacêuticas, em que essas células são utilizadas para simular a absorção de drogas pelo intestino humano após a ingestão (ARTURSSON; PALM; LUTHMAN, 2012).

Além do ramo farmacêutico, alguns estudos voltados para a engenharia de alimentos e nutrição têm mostrado grande eficiência desse modelo na simulação da absorção de elementos presentes em alimentos, como exemplo dos carotenoides acumulados em plantas. Os resultados de trabalhos que utilizaram células da linhagem Caco-2 para avaliar a biodisponibilidade de carotenoides, mostraram que esse método pode ser utilizado eficientemente para predizer a porcentagem que é absorvida desses nutrientes pelo organismo humano (CHITCHUMROONCHOKCHAI; SCHWARTZ; FAILLA, 2004; LIU; GLAHN; LIU, 2004; REBOUL et al., 2005).

Depois de absorvidos pelos enterócitos intestinais, os carotenoides são incorporados nos quilomicrons, os quais são eventualmente entregues à corrente sanguínea e, por fim, armazenados no fígado, onde podem permanecer armazenados ou transferidos para lipoproteínas de baixa ou alta densidade antes de serem depositados em algum tecido específico, no qual realizarão suas funções (ZARIPHEH; ERDMAN JÚNIOR, 2002). Saber o quanto de um determinado carotenoide presente em uma determinada planta é absorvido pelas células Caco-2, proporciona o acesso à real porcentagem da concentração inicial que está, de fato, disponível para utilização no metabolismo humano (AHERNE et al., 2010; GARRETT; FAILLA; SARAMA, 2000).

3 CONSIDERAÇÕES GERAIS

O presente trabalho está dividido em dois artigos, sendo o primeiro um estudo da identificação de genes que atuam na síntese e degradação de carotenoides em folhas de diferentes genótipos de alface e o segundo, um estudo da bioacessibilidade e biodisponibilidade de carotenoides presentes em genótipos de alface, bem como estudo do efeito do cozimento na liberação desses carotenoides durante a digestão *in vitro*.

No primeiro artigo, os resultados indicaram possíveis genes com capacidade para serem utilizados como marcadores para seleção de plantas de alface com altos níveis de clorofila total, luteína e β-caroteno, além de fornecer resultados que podem servir de base para estudos funcionais de avaliação da síntese e acúmulo de carotenoides em vegetais folhosos.

No segundo artigo, os resultados mostraram que diferenças nas concentrações de β-caroteno e luteína entre genótipos de alface, tanto em folhas novas quanto velhas, não estão relacionadas com a absorção e transporte desses carotenoides pelo intestino humano. O ato de cozinhar folhas de alface é capaz de romper a matriz alimentar das folhas, resultando em redução na concentração de carotenoides que seriam transferidos para a fração micelar após a digestão, mas essa redução é compensada em cerca de oito vezes na quantidade de carotenoides que passam para o interior do epitélio intestinal. A cultivar de alface Dark Land foi a mais eficiente juntamente as folhas externas de plantas da cultivar Salinas 88, em disponibilizar luteína e β-caroteno para absorção após o cozimento. Nesse sentido, os resultados propõem que programas de melhoramento genético para biofortificação de plantas devem ser conduzidos juntamente a outras linhas que visem ao aumento da biodisponibilidade dos carotenoides presentes na matriz alimentar.

REFERÊNCIAS

ÁGUILA RUIZ-SOLAA, M.; RODRÍGUEZ-CONCEPCIÓNA, M. Carotenoid Biosynthesis in Arabidopsis: A Colorful Pathway. **The Arabdopsis Book,** Rockville, v. 10, p. 28, 2012.

AHERNE , S. A. et al. Bioavailability of b-carotene isomers from raw and cooked carrots using an in vitro digestion model coupled with a human intestinal Caco-2 cell model. **Food Research International**, Essex, v. 43, p. 1449-1454, 2010.

AMPOMAH-DWAMENA, C. et al. Metabolic and gene expression analysis of apple (Malus x domestica) carotenogenesis. **Journal of Experimental Botany,** Oxford, v. 63, n. 12, p. 4497-511, July 2012.

AMPOMAH-DWAMENA, C. et al. The kiwifruit lycopene beta-cyclase plays a significant role in carotenoid accumulation in fruit. **Journal of Experimental Botany**, Oxford, v. 60, n. 13, p. 3765-79, 2009.

ANUÁRIO da agricultura brasileira. São Paulo: FNP, 2012. 137p.

ANUÁRIO da agricultura brasileira. São Paulo: FNP, 2014.

ARTURSSON, P.; PALM, K.; LUTHMAN, K. Caco-2 monolayers in experimental and theoretical predictions of drug transport. **Advanced Drug Delivery Reviews,** Amsterdam, v. 64, p. 280-289, 2012.

BOUIS, H. E. Micronutrient fortification of plants through plant breeding: can it improve nutrition in man at low cost? **Proceedings of the nutrition society,** London, v. 62, n. 2, p. 403-11, May 2003.

BUSTIN, S. A. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. **Journal of molecular endocrinology,** New York, v. 25, n. 2, p. 169-93, Oct. 2000.

CASSETARI, L. S. et al. β-carotene and chlorophyll levels in cultivars and breeding lines of lettuce. In: INTERNATIONAL SYMPOSIUM ON IN VITRO CULTURE AND HORTICULTURAL BREEDING, 8., 2013, Coimbra. **Anais…** Coimbra: Universidade de Coimbra, 2013. p. 181-181.

CAZZONELLI, C. I.; YIN, K.; POGSON, B. J. Potential implications for epigenetic regulation of carotenoid biosynthesis during root and shoot development. **Plant Signal Behavior**, Philadelphia, v. 4, p. 339-41, Apr. 2009.

CHITCHUMROONCHOKCHAI, C.; SCHWARTZ, S. J.; FAILLA, M. L. Assessment of lutein bioavailability from meals and a supplement using simulated digestion and caco-2 human intestinal cells. **Journal of Nutrition,** Philadelphia, v. 134, n. 9, p. 2280-6, Sept. 2004.

CLOTAULT, J. et al. Expression of carotenoid biosynthesis genes during carrot root development. **Journal of Experimental Botany,** Oxford, v. 59, p. 3563-73, 2008.

COURRAUD, J. et al. Stability and bioaccessibility of different forms of carotenoids and vitamin A during in vitro digestion. **Food Chemistry,** Barking, v. 136, n. 2, p. 871-7, Jan 15 2013.

DE PEE, S. et al. Lack of improvement in vitamin A status with increased consumption of dark-green leafy vegetables. **Lancet,** London, v. 346, n. 8967, p. 75-81, July 1995.

FAILLA, M. L.; HUO, T.; THAKKAR, S. K. In vitro screening of relative bioaccessibility of carotenoids from foods. **Asia Pacific journal of clinical nutrition,** London, v. 17, p. 200-203, 2008. Suppl. 1.

FARRARA, B. F.; ILOT, T. W.; MICHELMORE, R. W. Genetic analyses of factors for resistance to downey mildew (Bremia lactucae) in species of lettuce (Lactuca sativa and L. serriola). **Plant Pathology**, Oxford, v. 36, p. 499-514, 1987.

FILGUEIRA, F. A. R. **Novo manual de olericultura**: agrotcnologia moderna na produção e comercialização de hortaliças. Viçosa, MG: UFV, 2000. 402 p.

FOOD AGRICULTURE ORGANIZATION OF THE UNITED NATIONS. **Fao Statistical Yearbook**. 2013. Disponível em: <http://www.fao.org/docrep/018/i3107e/i3107e.PDF>. Acesso em: 22 jan 2015.

GACHON, C.; MINGAM, A.; CHARRIER, B. Real-time PCR: what relevance to plant studies? **Journal of Experimental Botany,** Oxford, v. 55, n. 402, p. 1445-54, July 2004.

GARRETT, D. A.; FAILLA, M. L.; SARAMA, R. J. Estimation of carotenoid bioavailability from fresh stir-fried vegetables using an in vitro digestion/Caco-2 cell culture model. **Journal of Nutritional Biochemistry**, Stoneham, 11, v. 11/12, p. 574-580, 2000.

GIBSON, U. E.; HEID, C. A.; WILLIAMS, P. M. A novel method for real time quantitative RT-PCR. **Genome Research,** New york, v. 6, n. 10, p. 995-1001, Oct. 1996.

GIULIANO, G.; BARTLEY, G. E.; SCOLNIK, P. A. Regulation of carotenoid biosynthesis during tomato development. **Plant Cell**, Rockille, v. 5, p. 379-87, Apr 1993.

GOMES, L. A. A.; MALUF, W. R.; CAMPOS, V. P. Inheritance of the resistance reaction of the lettuce cultivar 'Grand Rapids' to the southern root-knot nematode Meloidogyne incognita (Kofoid & White) Chitwood. **Euphytica,** Wageningen, v. 114, p. 37-46, 2000.

GRAHAM, R. D. et al. Breeding for micronutrient density in edible portions of staple food crops: conventional approaches. **Field Crops Research,** Amsterdam, v. 60, p. 57-80, 1999.

GRAHAM, R. D.; WELCH, R. M.; BOUIS, H. Addressing micronutrient malnutrition through the nutritional quality of staple foods: principles, perspectives, and knowledge gaps. **Advances in Agronomy**, San Diego, v. 70, p. 77-142, 2001.

HEDREN, E.; DIAZ, V.; SVANBERG, U. Estimation of carotenoid accessibility from carrots determined by an in vitro digestion method. **European journal of Clinical Nutrition**, London, v. 56, n. 5, p. 425-30, May 2002.

HEID, C. A. et al. Real time quantitative PCR. **Genome Research**, New York, v. 6, n. 10, p. 986-994, Oct. 1996.

HIGUCHI, R. et al. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. **Biotechnology**, New York, v. 11, n. 9, p. 1026-30, Sept. 1993.

INSTITUTO DE ECONOMIA AGRÍCOLA DO ESTADO DE SÃO PAULO. 2014. Disponível em: < http://www.iea.sp.gov.br/out/bancodedados.html>. Acesso em; jan 2015.

JOLLIFFE, F. D. The use of sensory evaluation and consumer preference for the selection of sweetpotato cultivars in East Africa. **Journal of the Science of Food and Agriculture,** Oxford, v. 84, p. 791-799, 2004.

KATO, M. et al. The role of carotenoid cleavage dioxygenases in the regulation of carotenoid profiles during maturation in citrus fruit. **Journal of Experimental Botany**, Oxford, v. 57, n. 10, p. 2153-64, 2006.

KISHIMOTO, S.; OHMIYA, A. Regulation of carotenoid biosynthesis in petals and leavesof chrysanthemum (Chrysanthemum morifolium). **Physiologia Plantarum**, Sweden, v. 128, p. 436–447, 2006.

LEBEDA A. et al. Lettuce (Asteraceae; Lactuca spp.). In: SINGH, R. J. (Ed.). **Genetic resources, chromosome engineering, and crop improvement. vegetable crops**. Boca Raton: CRC, 2007. v. 3, p. 377–472.

LEBEDA, A.; SCHWINN, F. J. The downey mildews - an overview of recent research progress. **Jounal of Plant Diseases and Protection**, Berlin, v. 101, p. 225-254, 1994.

LEMMENS, L. et al. Towards a better understanding of the relationship between the beta-carotene in vitro bioaccessibility and pectin structural changes: A case study on carrots. **Food Research International,** Essex, v. 42, p. 1323-1330, 2009.

LINDQVIST, K. On the origin of cultivated lettuce. **Hereditas,** Landskrona**,** v. 46, p. 319-350, 1960.

LIU, C. S.; GLAHN, R. P.; LIU, R. H. Assessment of carotenoid bioavailability of whole foods using a Caco-2 cell culture model coupled with an in vitro digestion. **Journal of agricultural and food chemistry,** Washington, v. 52, n. 13, p. 4330-4337, June 2004.

LOCKEY, C.; OTTO, E.; LONG, Z. Real-time fluorescence detection of a single DNA molecule. **Biotechniques**, Natick, v. 24, n. 5, p. 744-6, May 1998.

MELO, A. M. T.; MELO, P. C. T. Hiroshi Nagai (1935-2003): sua vida e contribuição à olericultura. **Horticultura Brasileira**, Vitória da Conquista, v. 21, 2003. Disponível em: < http://www.scielo.br/scielo.php?pid= S0102-05362003000400034&script=sci_arttext>. Acesso em: 23 dez. 2014.

MOU, B. Genetic Variation of Beta-carotene and Lutein Contents in Lettuce. **Journal of the American Society for Horticultural Science**, Alexandria, v. 130, p. 7, 2005.

MULLIS, K. B.; FALOONA, F. A. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. **Methods Enzymology,** New York, v. 155, p. 335-50, 1987.

NAGAI, H. Obtenção de novos cultivares de alface (Lactuca sativa L.) resistentes ao mosaico e ao calor. I-Brasil 48, 202 e 221. **Revista de Olericultura**, Campinas, v. 17, p. 129-137, 1979.

NAGAO, A. Bioavailability of Dietary Carotenoids: Intestinal Absorption and Metabolism. **JARQ - Japan Agricultural Research Quarterly**, Tokyo, v. 48, p. 385-391, 2014.

NESTEL, P. et al. Biofortification of staple food crops. **Journal of Nutrition,** Philadelphia, v. 136, n. 4, p. 1064-1067, Apr. 2006.

OBRERO, Á. et al. Carotenogenic gene expression and carotenoid accumulation in three varieties of Cucurbita pepo during fruit development. **Journal of agricultural and food chemistry**, Washington, v. 61, p. 6393-6403, July 2013.

PAINE, J. A. et al. Improving the nutritional value of Golden Rice through increased pro-vitamin A content. **Nature Biotechnology,** New York, v. 23, n. 4, p. 482-487, Apr. 2005.

REBOUL, E. et al. Enrichment of tomato paste with 6% tomato peel increases lycopene and beta-carotene bioavailability in men. **Journal of Nutrition,** Philadelphia, v. 135, n. 4, p. 790-794, Apr. 2005.

RICH, G. T. et al. Solubilization of carotenoids from carrot juice and spinach in lipid phases: I. Modeling the gastric lumen. **Lipids,** Champaign, v. 38, n. 9, p. 933-45, Sep 2003.

RYAN, L. et al. Micellarisation of carotenoids from raw and cooked vegetables. **Plant Foods for Human Nutrition**, Dordrecht, v. 63, n. 3, p. 127-33, Sept. 2008.

RYDER, E. J. A mild systemic reaction to lettuce mosaic virus in lettuce: Inheritance and interaction with an allele for resistance. **Journal of the American Society for Horticultural Science**, Alexandria, v. 127, p. 814-818, 2002.

SALA, F. C.; COSTA, C. P. D. Retrospective and trends of Brazilian lettuce crop. **Horticultura Brasileira**, Vitória da Conquista, v. 30, p. 187-194, 2012.

SALA, F. C. Melhoramento genético de alface. In: CONGRESSO BRASILEIRO DE OLERICULTURA, 51, 2011, Viçosa, MG. **Anais...** Viçosa, MG: ABH, 2011. p. 5813-5827.

SOUSA, C. S. D. et al. Genetics divergence among lettuce genotypes by AFLP markers. **Bragantia**, Campinas, v. 66, p. 11-16, 2007.

VAN HET HOF, K. H. et al. Dietary factors that affect the bioavailability of carotenoids. **Journal of Nutrition**, Rockville, v. 130, n. 3, p. 503-506, Mar. 2000.

ZARIPHEH, S.; ERDMAN JÚNIOR, J. W. Factors that influence the bioavailablity of xanthophylls. **Journal of Nutrition,** Rockville, v. 132, n. 3, p. 531-534, Mar. 2002. Suppl.

ARTIGO 1

IDENTIFICATION OF CAROTENOID BIOSYNTHETIC PATHWAY GENES WHOSE TRANSCRIPT LEVELS ARE CORRELATED WITH CAROTENOID LEVELS IN LETTUCE (*Lactuca sativa*)

Artigo redigido nas normas da revista "Jounal of Experimental Botany"

ABSTRACT

Green leafy vegetables containing health-promoting carotenoids have potential for improving human health and nutrition. Among the green leafy vegetables, lettuce (*Lactuca sativa* L.) is the most important worldwide. There are a number of different types of lettuce cultivars which exhibit differences in leaf color and plant architecture. While expression levels of carotenoid pathway genes have been correlated with carotenoid metabolite levels in some plants, there is a lack of studies to identify genes whose expression levels at seedling stage can be correlated with levels of β-carotene and lutein at the harvested final plant stage. In this study, we investigated the transcript levels of seventeen genes involved in carotenoid biosynthesis pathway during plant development in young and mature leaves across six lettuce cultivars that displayed visible variation in green color intensity of their leaves. Significant variation in carotenoid concentration between leaf age and cultivars was identified. The accumulation of β-carotene increased across the plant developmental life cycle until 40 days after sowing, and had decreased at commercial size stage 60 days after sowing. In contrast, lutein levels do not significantly vary during plant development. Correlation analyses identified carotenoid biosynthetic pathway genes whose expression levels could be used as biomarkers for prediction of total chlorophyll, lutein and β-carotene levels in final commercial stage lettuce plants. In this regard,

GGPPS, *PSY1* and *LCY-β1* were significant in predicting final carotenoid accumulation in commercial lettuce plants, while *CRTISO* and *LCY-β2* expression at the commercial size stage was correlated with carotenoid accumulation in this time. This study provides candidate gene biomarkers for selection of lettuce plants with high levels of total chlorophyll, lutein and β-carotene levels, and provide a framework for functional studies to investigate the synthesis and accumulation of carotenoids in green leafy vegetables.

Keywords: Carotenoids, carotenoid synthesis, gene expression, *Lactuca sativa*, lettuce, lettuce development.

1 INTRODUCTION

Dark green leafy vegetables are under consideration as sources of β-carotene and lutein to improve human health (Adefegha and Oboh, 2013; de Pee *et al.*, 1995; Raju *et al.*, 2007). Understanding the relationship between expression levels of carotenoid biosynthesis genes and the accumulation levels of β-carotene and lutein in leafy vegetable species is important for plant breeding and/or metabolic engineering approaches for altering such health-associated metabolites in leafy vegetable cultivars. A number of studies have compared gene expression levels with carotenoid accumulation across the plant life cycle and in different organ parts. Such studies have been conducted in apple (Ampomah-Dwamena *et al.*, 2012), tomato (Giuliano *et al.*, 1993), cabbage (Tuan *et al.*, 2012), courgette (Obrero *et al.*, 2013), squash (Nakkanong *et al.*, 2012), carrot (Clotault *et al.*, 2008) chrysanthemum (Kishimoto and Ohmiya, 2006) and kiwifruit (Ampomah-Dwamena *et al.*, 2009).

Differences in concentrations of lutein and beta-carotene in different genotypes (cultivars) of green leafy vegetable crops have been described, and

genetic variation underlying carotenoid concentrations has revealed significant correlations with chlorophyll concentration/green color intensity in lettuce (Mou, 2005), kale (Kopsell *et al.*, 2004) and sweet basil (Kopsell *et al.*, 2005). However, there is an absence of studies on the relationship between expression levels of carotenoid pathway genes and carotenoid levels in green leafy vegetables.

Accumulation of carotenoids usually occurs in plastids (e.g. chromoplasts), within tissues that accumulate the highest amount (e.g. roots, tubers, flowers and fruits), resulting in typical orange and yellowish colors of different plant organs. In photosynthetic tissues, carotenoid accumulation occurs in the chloroplasts, where they act as light harvesting and protectors against photooxidative damage to chlorophylls (Águila Ruiz-Solaa and Rodríguez-Concepcióna, 2012; Cazzonelli *et al.*, 2009). In animals, carotenoids are important dietary health-related compounds, acting as antioxidants preventing oxidative damage to cells, mainly to avert and relieve some age-related eye diseases (Fraser and Bramley, 2004).

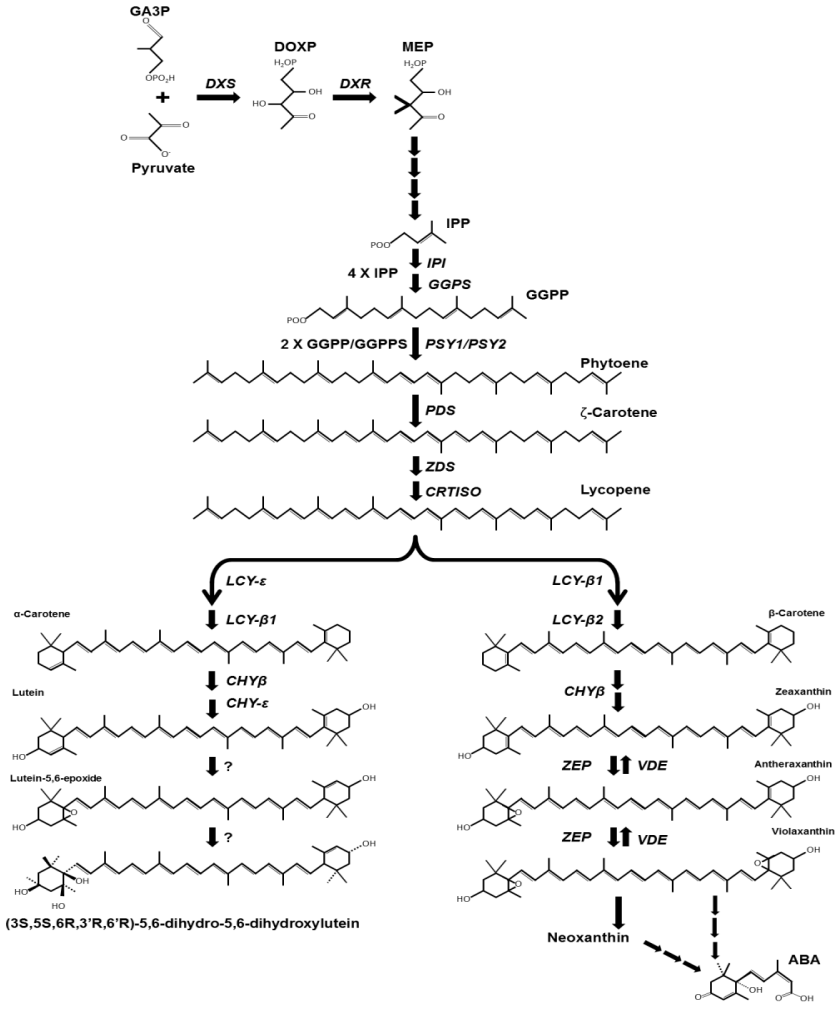
Beta-carotene is the most important pro-vitamin A carotenoid, being cleaved to vitamin A after uptake to the human body (Yeum and Russell, 2002). Undernutrition in relation to vitamin A intake is a major problem in developing countries and can result in permanent blindness and increase the extent of susceptibility to diseases (West, 2002; West and Darnton-Hill, 2001). Lutein is a xanthophyll carotenoid and is the most prevalent carotenoid accumulated in the retina of the human eye. The high concentrations of this carotenoid in the retina increases the extent of photo-protection, reducing by more than 80% the risks of age-related macular degeneration (Bone *et al.*, 2001; Khachik *et al.*, 1997).

There are more than 700 types of carotenoids identified in photosynthetic organisms. The biosynthesis of carotenoids in higher plants starts from C5-isopentenyl pyrophosphate (IPP), an isoprene unit where four molecules are condensed in one C20-geranylgeranyl diphosphate (GGPP). The GGPP is converted to phytoene by the enzyme phytoene synthase (PSY). Phytoene is subsequently

degraded to lycopene via ζ-carotene (first yellow carotenoid) by the enzymes phytoene desaturanse (PDS) and ζ-carotene desaturase (ZDS). The ends of the linear carotenoid lycopene can then be cyclized by lycopene β-cyclase (LCY-β1) and/or lycopene ε-cyclase (LCY-ε), and the molecule is modified to express a variety of structural features by hydroxylation. The combined action of the two previous enzymes allows synthesis of α-carotene from lycopene, while the single action of LCY-β leads to β-carotene. After synthesis, α-carotene can then be hydroxylated into lutein by both β-ring hydroxylase (CHYβ) and α-ring hydroxylase (CHYε), and β-carotene is hydroxylated to zeaxanthin by CHYβ. Zeaxanthin is then transformed into violaxanthin via antheraxanthin by zeaxanthin epoxidase (ZEP) (Fig. 1).

A range of molecular mechanisms regulate carotenoid biosynthesis and accumulation in the plastids during plant development. Differences in the transcript levels of genes involved in carotenoid metabolism can directly be related to the extent of carotenoid synthesis or degradation. In apple, carotenoid accumulation in the harvested ripe fruits can be predicted during fruit development by increased levels of *ZISO*, *CRTISO*, and *LCY-ε* transcripts (Ampomah-Dwamena *et al.*, 2012). Similar predictive relationships have been found in carrot, whose the increase of transcript levels of *LCY-ε* was correlated with lutein accumulation, while *ZDS1* and/or *ZDS2* transcript levels were correlated with accumulation of lycopene (Clotault *et al.*, 2008).

Figure 1 The asteraceae carotenoid biosynthetic pathway.



Starting from the isoprene, the arrows indicate the enzymatic conversions. Abbreviations: GA3P, glyceraldehyde-3-phosphate; DXS, 1-deoxyxylulose 5-phosphate synthase; DOXP, D-1-deoxyxylulose-5-phosphate; DXR, 1-deoxyxylulose 5-phosphate reductoisomerase; MEP, 2-C-methyl-D-erythritol-2,4- cyclodiphosphate; IPP, isopentenyl diphosphate; IPI, IPP isomerase; GGPP, geranylgeranyl diphosphate; GGPS, GGPP synthase; PSY1, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LCY-B, lycopene β-cyclase; LCY-E, lycopene ε-cyclase; CHYB, β-ring hydroxylase; CHYE, ε-ring hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin deepoxidase.

The presence of chlorophyll-binding proteins and lipoproteins that sequester carotenoids in plastids can also affect the accumulation of carotenoids in plants (Vishnevetsky *et al.*, 1999). Source-sink relationships affecting flux of metabolites in biosynthetic pathways are another factor that affect carotenoid accumulation in plants(Cazzonelli and Pogson, 2010). In fruits, the conversion of chloroplasts to chromoplasts during fruit development coincides with the biosynthesis of carotenoids, making chromoplasts the major storage structures of these metabolites in ripe fruits. However, in green leafy vegetables such conversions do not occur, and the plant forms carotenoid crystals to increase the accumulation capacity (Maass *et al.*, 2009a). A similar mechanism has been identified in cauliflower where plastid differentiation is affected by a mutant gene *Or,* which causes β-carotene accumulation in plant curds and increases the sink capacity (Lu *et al.*, 2006). The degradation of carotenoids produced at the end of the pathway is another factor that affects carotenoid concentrations in plants. Degradation mechanisms involve the activity of a group of enzymes, the carotenoid cleavage dioxygenases (CCDs). For instance, white-flowered chrysanthemum cultivars have higher levels of *CmCCD4a* transcript in their petals comparing to yellow-flowered cultivars. After the introduction of an RNAi construct of *CmCCD4a*, the white petals turned yellow, indicating that the carotenoids are synthesized but are subsequently degraded into colorless compounds, which results in the white color (Ohmiya *et al.*, 2006).

From a nutritional perspective, lettuce cultivars display a large variation in several nutritional components that are potentially beneficial to human health and this variation can be exploited to improve nutritional quality (Hayashi *et al.*, 2012). Depending on cultivar, lettuce contains 20–80% of the antioxidant content of red cabbage and 30–160% of that of spinach, showing a huge variation to carotenoid concentration along different cultivars. The most widely consumed

lettuce, the iceberg type, shows the least amount of antioxidant activity of the main lettuce cultivars (Llorach *et al.*, 2008; Mou, 2005; Wu *et al.*, 2004).

In this study, we analyzed the carotenoid composition and concentration in inner and outer leaves along the plant life cycle for six contrasting lettuce cultivars selected for contrasting leaf color and plant architecture. We also investigated the correlations between transcript levels of carotenoid biosynthesis pathway genes and accumulation of specific carotenoids in leaves. Our study identified carotenoid biosynthetic genes that can be used as seedling stage biomarkers for prediction of β-carotene and lutein levels in commercial size lettuce plants from different lettuce cultivars.

2 MATERIAL AND METHODS

2.1 Plant material and growing conditions

Six commercial lettuce cultivars were chosen for analysis based on their differences in plant architecture and color intensity: Dark Land Cos MT (romaine type, dark green), Dragoon (mini romaine, dark green), Grand Rapids (batavia type, light green), Parris Island (romaine type, medium green), Salinas 88 (crisphead, medium green intensity) and Verônica (batavia, light green). Plants were grown at the National University of Ireland Galway, between February and March 2014, in a plant growth chamber under 16 hours day light period. Lettuce seeds were sown on soil in 200 mL pots and transplanted to three liter pots at seedling size (20 days after sowing). The experiments were conducted in a complete randomized design with three replications and three plants per plot. All plants were grown at the same time and in the same chamber, with the same light intensity and temperature to minimize environmental variability. Leaf samples of each cultivar were harvested at three different stages, corresponding to seedling size (transplanting time), 40 days after sowing (DAS), and at commercial size (60 days after sowing), using one different group

of plants per harvest. At the 40 and 60 DAS stages, the outer leaves (first external leaves) and inner leaves (a leaf from the seventh inner layer of leaves from the outside of the head) were sampled. In each lettuce plant, sampling was done on three different leaves around the plant in the same location in each outer or inner leaf, corresponding to a total of three pooled samples per plant, and nine pooled samples per plot. The harvested samples from each sampling time and leaf stage were frozen immediately and stored at -80º C until analysis.

2.2 Carotenoid extraction and HPLC analyses

Carotenoid pigments were extracted following the procedures described by Norris with some modifications (Norris *et al.*, 1995). Leaf tissue (0.3 g) was ground under liquid nitrogen in a porcelain mortar and transferred to a 2 mL centrifuge tube with one glass bead. 200 µL of 80% v/v acetone was added before adding ethyl acetate (200 µL), and the tubes were agitated at 30,000 rpm for 1 min in a tissuelyser (RETSCH MM200 – Qiagen, Manchester, UK). Water (140 µL) was added and the mixture was agitated again at 30,000 rpm for 1 min and then centrifuged at 15.800g_n for 5 min in a microcentrifuge (Heraeus Fresco 17 – Thermo Scientific, Dublin, Ireland). The carotenoid containing upper phase was then transferred to a new tube. The samples were extracted at least three more times, adding 200 µL of ethyl acetate, agitating, centrifuging at 15.800g_n for 5 min, and removing the upper phase until the leaf sediments did not have any visible green color. The combined ethyl acetate phases were vacuum dried in a centrifugal evaporator (miVac – GeneVac SP Scientific, Ipswich, UK). The dried samples were subsequently redissolved in 1.5 mL of 0.8% of BHT/acetone (Ampomah-Dwamena *et al.*, 2009) and analyzed by the reverse phase of high performance liquid chromatography (HPLC). The HPLC system (Alliance, Waters Co., Milford, Mass.) consisted of a separation unit (model 2695), YMC 4.6 x 10 mm C30 guard cartridge and YMC RP C30 column (3 µm, 250 × 4.6 mm - YMC, Wilmington, North Carolina, USA). The column temperature was 25 °C and samples

were kept in 4 ºC sample cooler and a 50 µL aliquot was injected into a 1 mL min^{-1} flow rate. The elution was performed using a mobile phase comprising solvent A (MeOH), solvent B [H2O/MeOH, 20:80) containing 0.2% w/v ammonium acetate], and solvent C (*tert*-butyl methyl ether). The elution gradient was a time reduced version of that described by Ampomah-Dwamena *et al.* (2012). The gradient started with 95% A/5% B for 2 min, decreasing to 80% A/5% B/15% C between 2 and 10 min, decreasing to 30% A/5% B/65% C by 15 min, decreasing to 25% A/5% B/70% C at 20 min, and returning to 95% A/5% B at 25 min. β-Carotene and lutein were identified by comparing the retention time and absorption spectra of individual peaks with the standards. The β-Carotene, lutein and the internal standard *trans*-β-Apo-Carotenal were purchased from Sigma Aldrich (Arklow, Wicklow, Ireland). The concentrations of both β-Carotene and lutein were determined as µg per 100g of fresh leaves, considering extraction losses determined from the initial concentration of the internal standard.

2.3 Chlorophyll extraction and quantification

Chlorophyll pigments were extracted by crushing the samples in a tissuelyser (RETSCH MM200 – Qiagen, Manchester, UK) in tubes with one glass bead for 1 min at 30,000 rpm. 2 mL of 96% v/v ethanol was added and the samples were agitated again for 1 min at 30,000 rpm and left for 24 hours at 4 ºC in a 2 mL centrifuge tube. After this time period, the samples were submitted for absorbance measurement in a spectrophotometer (Nanophotometer – Inplen, München, Germany) at 649 and 665 nm wavelengths. The chlorophyll a (C_a) and b (C_b) concentrations were by the following formulas (Lichtenthaler and Wellburn, 1983):

$$C_a = 13.95A_{665} - 6.88A_{649}$$

$$C_b = 24.96A_{649} - 7.32A_{665}$$

The total chlorophyll was calculated by the sum of C_a and C_b. and the values were determined as µg $100g^{-1}$ of fresh leaves.

2.4 RNA extraction and cDNA synthesis

Total RNA was extracted from leaf samples using the *Isolate II RNA Mini Kit* (Bioline, London, UK). Samples were crushed with glass beads in a tissulyser (RETSCH MM200 – Qiagen, Manchester, UK) for 1 min at 30,000 rpm. Lysis buffer was added to the ground tissue followed by vigorous vortexing. The lysate was loaded in the filter tube column and centrifuged for 1 min at 11,000*g* (Heraeus Fresco 17 – Thermo Scientific, Dublin, Ireland) to isolate impure particles. In the filtered samples 70% v/v ethanol was added, followed by vortexing and loading in a second filter tube column. The samples were centrifuged for 30s at 11,000*g* and washed two times with wash buffer and dried. Total RNA was resuspended in RNase-free water and treated with DNase. cDNA was synthesized from total RNA (0.5–1 µg) using a SensiFAST cDNA Synthesis kit (Bioline, London, UK) following the manufacturer's protocol. The reaction components were 5x TransAmp Buffer and Reverse Transcriptase. The reaction was incubated at 25 °C for 10 min (primer annealing), 42 ºC for 15 min (reverse transcription) and 85 ºC for 5 min (inactivation).

2.5 Quantitative real-time PCR analysis

Lettuce genes involved in carotenoid biosynthetic pathway were identified through searchers of the Expressed Sequence Tag (EST) database deposited in NCBI (National Center for Biotechnology Information) using tBlastn tool and using as queries known carotenoid biosynthetic proteins from chrysanthemum, apple and *Arabidopisis thaliana*. For the seventeen target carotenogenic genes and the ubiquitin housekeeping gene

(Supplementary Table S1), primers were designed using Quantprime (http://www.quantprime.de/main.php?page=home). Quantitative real-time PCR (qRT-PCR) was performed using a CFX96 Real-Time system (BioRad, Hemel Hempstead, Hertfordshire, UK). The SYBR Green master mix was used following manufacturer's protocol with minimum modifications. cDNA templates were diluted 1:4 times and used in a 5 µL final volume reaction (1 µL of cDNA template, 0.25 µL of each primer, 1 µL of water and 1 µL of SYBR green). For each sample three technical replicates were prepared with three negative controls per plate. The PCR reaction conditions were 95 °C for 10 min (preincubation), followed by 40 amplification cycles (95 ºC for 15 s, 60 ºC for 30 s and 72 ºC for 30 s). A melting curve analysis with continuous fluorescence measurement during the 65–95 °C melt was generated after the amplification reactions. Data were analysed using BioRad CFX manager software (BioRad, Hemel Hempstead, Hertfordshire, UK). The expression level of each gene was normalized to the *Lactuca sativa* Ubiquitin gene, which was used as the housekeeping gene. Standard deviation was calculated using the three biological repetitions for each sample.

2.6 Correlation analyses between transcript levels and carotenoids content

The transcript levels of each gene as measured by qRT-PCR in seedling, 40 and 60 DAS tissues (including inner and outer leaves) were correlated with β-carotene and lutein content. A Pearson correlation (*r*) analysis was performed and tested for statistical significance using the "proc corr" procedure in SAS Statistical Analysis Software (www.sas.com).

3 RESULTS

Commercial size lettuce plants contain a range of lutein, β-carotene and chlorophyll levels (Mou, 2005). In other green vegetables such as kale (Kopsell *et*

al., 2004) and sweet basil (Kopsell *et al.*, 2005) differing carotenoid and chlorophyll levels have also been reported, while in chinese cabbage a correlation between carotenoid accumulation and expression levels of carotenoid biosynthesis genes has been reported (Tuan *et al.*, 2012). However, there have been no systematic studies to investigate the expression levels of carotenoid biosynthesis genes and carotenoids during lettuce plant development. To determine the carotenoid content of different cultivars of lettuce plants at different developmental stages, reverse phase HPLC was used.

3.1 Analysis of lutein, β-carotene and chlorophyll levels in commercial size lettuce plants

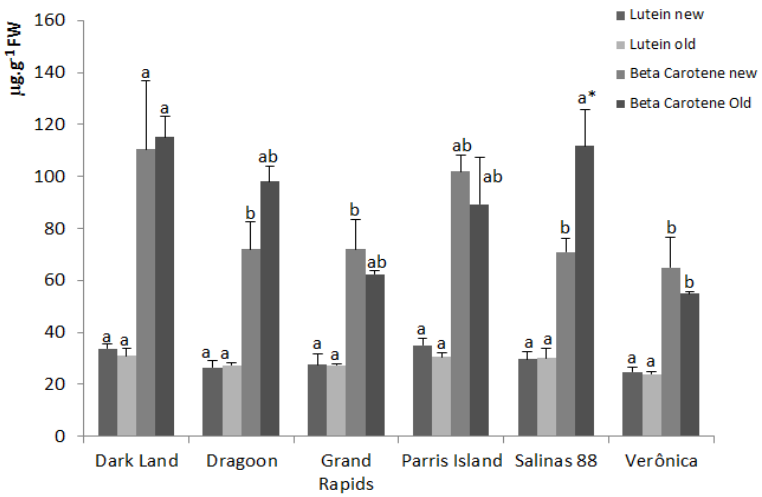
Carotenoid content was determined in lettuce plants at commercial size in inner and outer leaves across six lettuce cultivars which displayed extreme variation in leaf color (Fig. 2 and Fig. 3 lower panel). In addition, chlorophyll concentrations were analysed by absorbance wavelength in a spectrophotometer (Table 1).

At the commercial size stage the cultivar Dark Land had the highest lutein and β-carotene concentrations in both outer and inner leaves, wherein the β-carotene concentrations were almost three fold those observed for lutein (Fig. 2). The light green cultivars Grand Rapids and Verônica had the lowest concentrations of both carotenoids and a small significant difference was observed to β-carotene between inner and outer leaves, with inner leaves displaying a higher concentration. Conversely, a higher concentration of β-carotene in outer leaves was observed in 'Dragoon' and 'Salinas 88', both genotypes with a differential color between outer and inner leaves due the arrangement of leaves in plant. The conformation and shell shape of the outer leaves of these cultivars tended to hide the inner leaves from light resulting in inner light green leaves (Fig. 3). In the case of 'Dark Land' and 'Parris Island' it was found that they had equal concentrations of β-carotene in inner and outer

leaves (Fig. 2). These are both romaine type cultivars with all leaves exposed to the same light intensity (Fig. 3).

In lettuce our results indicate that green color intensity is a good indicator of β-carotene concentration, with dark green leaves accumulating more β-carotene than light green ones. This is consistent with other studies screening levels of β-carotene and lutein in lettuce germplasm (Mou, 2005). Although total chlorophyll is correlated with β-carotene concentration ($R^2 = 0.8$, $P < 0.05$), the lutein concentration in lettuce has no relationship with leaf color ($P > 0.05$). Even though 'Dragoon' and 'Salinas 88' had different leaf shape and color between inner and outer leaves, these cultivars had the same concentration of lutein in the inner and outer leaves. This suggests that lutein concentrations are unrelated to leaf color intensity.

Figure 2 Lutein and β-carotene concentration in inner and outer leaves of lettuce cultivars as measured by HPLC analysis.



Plants were harvested at commercial size and separated into inner and outer leaves for carotenoid extraction and analysis. Error bars are standard deviation of the mean from three biological replicates, each one being the pooled sample of at least three technical replicates, at $P = 0.05$ level. Bars with similar letters for lutein and β-carotene in each leafy position are not significantly different ($P = 0.05$), using one-way ANOVA analysis followed by Tukey pos hoc test. The * means significative difference between inner and outer leaves.

Figure 3 Lettuce plants development series of cultivars used in carotenoid analysis. Plants were harvested from six genotypes growing under the same environmental conditions, at seedling, 40 days after sowing (DAS), and at commercial size stage.

Table 1 Chlorophyll concentrations (μg.g $^{-1}$ fresh weight) in inner and outer leaves of commercial size lettuce plants of different cultivars

Chorophyll[1]	Dark Land	Dragoon	Grand Rapids	Parris Island	Salinas 88	Verônica
Inner leaves						
Chl *a*	653.17	666.93	402.92	1144.59	307.58	254.98
Chl *b*	448.81	388.31	287.23	586.96	135.70	116.62
Chl Total	1101.98	1055.24	690.15	1731.55	443.29	371.61
Chl *a*/Chl *b*	1.46	1.72	1.40	1.95	2.27	2.19
Outer leaves						
Chl *a*	1211.49	1470.75	346.61	1217.21	1304.82	596.47
Chl *b*	575.05	743.15	152.89	646.33	485.14	353.37
Chl Total	1786.54	2213.90	499.51	1863.54	1789.97	949.84
Chl *a*/Chl *b*	2.11	1.98	2.27	1.88	2.69	1.69

[1] Measured by spectrophotometer absorbance.

Values represent the average of three biological replicates, each one being the pooled sample of at least three technical replicates.

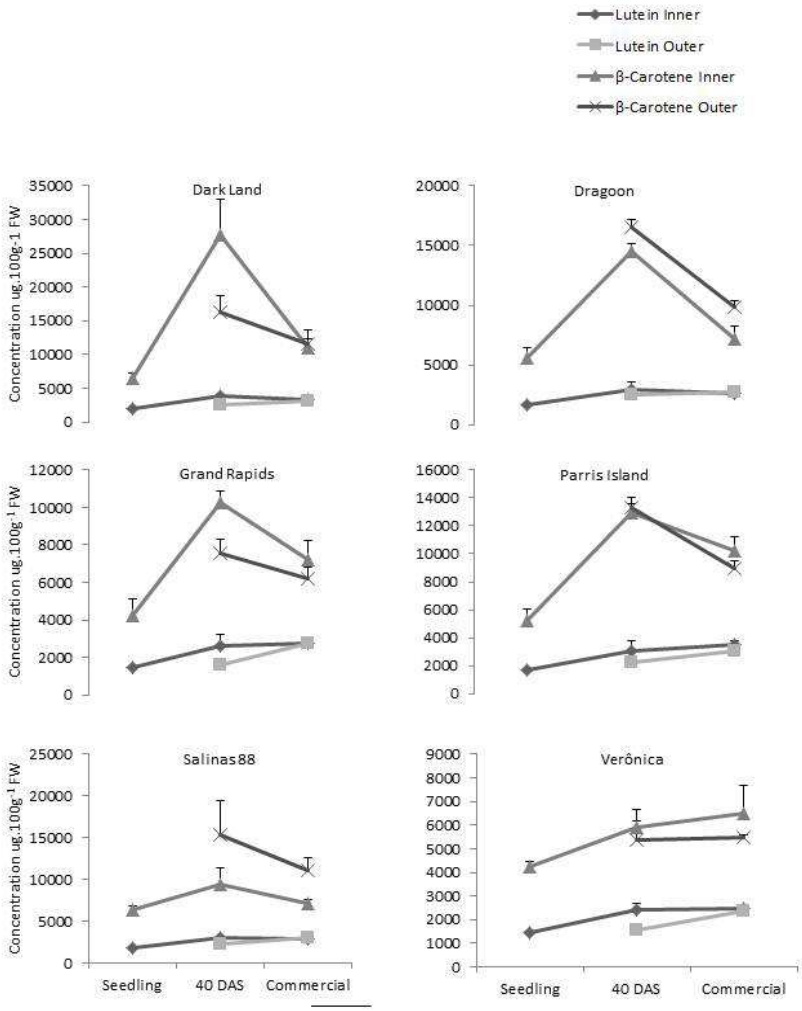
The profile of chlorophyll *a*, *b* and total chlorophyll displayed similarities to the profile of carotenoids along plant development. At commercial size, the different lettuce cultivars had different chlorophyll concentrations in inner and outer leaves (Table 1). In all genotypes, the concentration of chlorophyll *a* was higher than chlorophyll *b* in both leaf ages. The concentration of chlorophyll total was higher in outer leaves, except for the cultivar Grand Rapids which had less chlorophyll in outer leaves than in inner ones.

3.2 β-carotene and lutein accumulation during lettuce plant development

Three different stages of lettuce plant development were sampled across six contrasting lettuce cultivars and analysed for β-carotene and lutein concentrations (Fig. 3). The concentration of β-carotene was higher than lutein for all cultivars across all samplings (Fig. 4). The light green cultivars Grand Rapids and Verônica had lower concentrations of β-carotene and lutein across the developmental stages tested. At seedling size, all lettuce cultivars exhibited low concentrations of β-carotene and lutein, with increases in accumulation observed during later plant development.

Lutein concentrations increased most evidently from the seedling size to 40 DAS for all lettuce cultivars, while from the 40 DAS stage onwards there was no major increase observed. In the case of β-carotene, while there were major increases in levels from the seeding to 40DAS stage, in many instances there was a reduction in levels at commercial stage size (Fig 4 and Supplementary Tables S2 and S3). This trend was observed for both of the carotenoids for all cultivars in inner and outer leaves. At 40 DAS sampling the highest concentration of β-carotene was found in inner leaves, except in the cultivars Dragoon and Salinas 88. The lower concentration of β-carotene in the inner leaves of those two cultivars could be due the plant architecture, as the inner leaves were not exposed to light at the 40 DAS sampling time (Fig. 3).

Figure 4 β-carotene and lutein content patterns during plant development in six lettuce cultivars. Error bars are the standard deviation of the mean from three biological replicates.



At the commercial stage sampling, the ranking of β-carotene between inner and outer leaves in each of these two cultivars remained the same, with internal leaves hidden in function of the plant architecture that had a tendency to close the heads in commercial stage (Fig. 2 and Fig. 4). The other four cultivars retained a higher concentration of β-carotene in inner leaves.

3.3 Identification of carotenoid biosynthetic pathway genes in lettuce

To determine whether carotenoid accumulation during lettuce development (i.e. different leaf ages and locations), as well as whether differences in carotenoid composition between lettuce cultivars could be related to the expression level of carotenoid biosynthetic genes, transcript levels of seventeen carotenoid biosynthesis genes in lettuce were analysed by qRT-PCR (Table 1), using the same samples used in the carotenoid and chlorophyll quantification (Fig. 5 and Fig. 6). To identify genes involved in carotenoid accumulation in lettuce, tBLASTn tool and reference proteins from chrysanthemum, apple and *A. thaliana* were used as queries and searches against lettuce EST databases (NCBI) and genome project sequence (https://lgr.genomecenter.ucdavis.edu/Links.php). In the published lettuce genome, low gene copy numbers for a number of these genes were found i.e. two copies of geranylgeranyl diphosphate (*GGPS* and *GGPPS*) were identified, along with two copies of phytoene synthase (*PSY1* and *PSY2*) and two copies of lycopene β-cyclase (*LCY-β1* and *LCY-β2*).

Figure 5 Expression of lettuce carotenoid biosynthetic genes in inner leaves of different lettuce cultivar measured at seedling, 40 days after sowing (DAS), and commercial size stage plants. Gene expression was measured relative to *ubiquitin.* Error bars are standard deviation of the mean from 3 biological replicates.

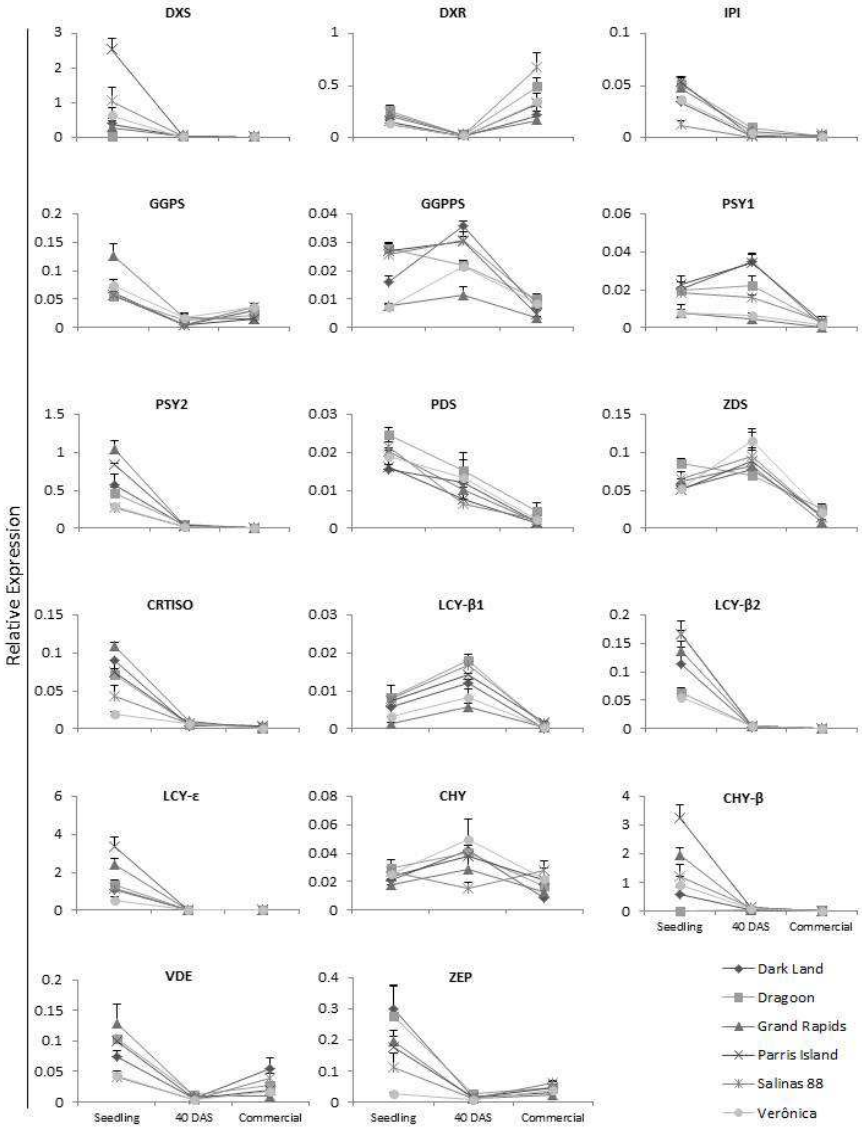
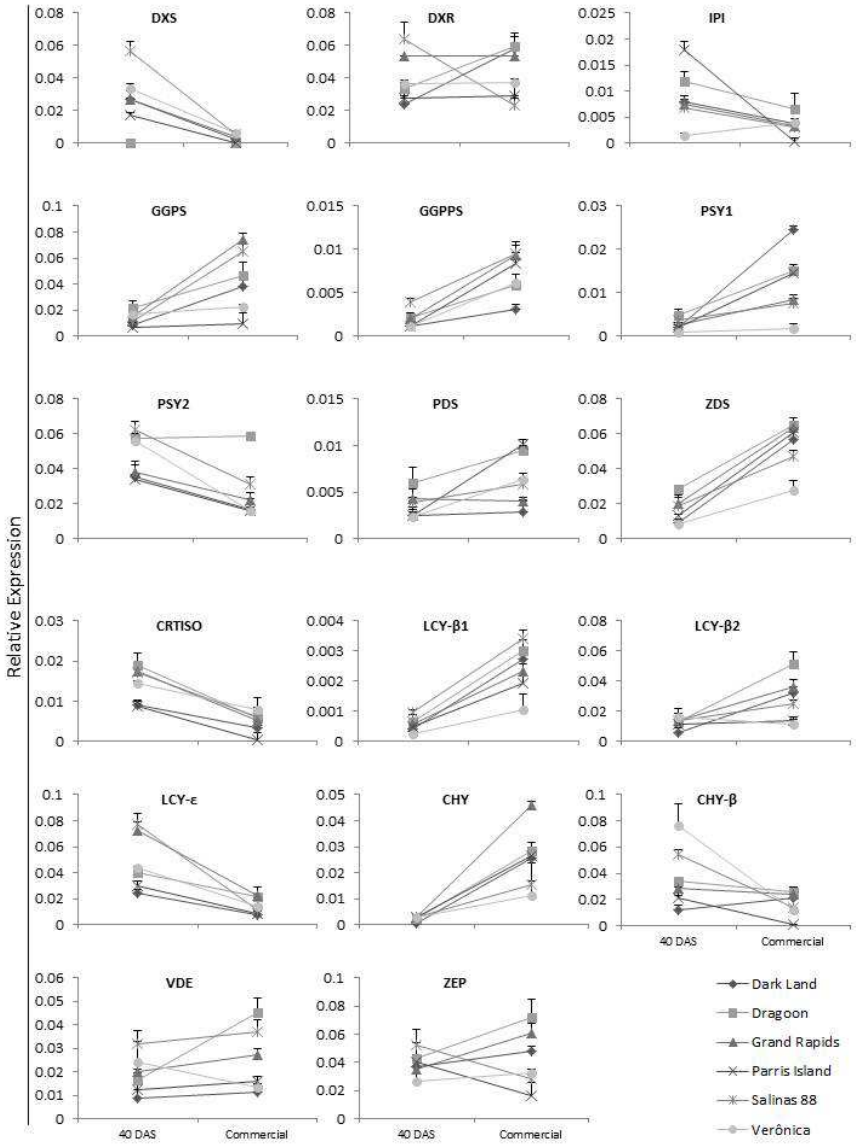


Figure 6 Expression of lettuce carotenoid biosynthetic genes in outer leaves of different lettuce cultivar measured at 40 days after sowing (DAS) and commercial size plants. Gene expression was measured relative to *ubiquitin*. Error bars are standard deviation of the mean from 3 biological replicates.



3.4 Transcript levels of carotenoid biosynthetic pathway genes during lettuce plant development

The gene expression profiles and levels of carotenoid biosynthesis genes during plant development were analyzed. Transcripts of all of the carotenoid biosynthesis genes tested were detected in all samples, indicating that expression of these genes occurs between seedling and commercial size, including in the light green cultivars Grand Rapids and Verônica and the dark green cultivars, regardless the leaf age (Fig. 5 and Fig. 6). Overall, the levels of transcript were highest in the inner leaves compared with outer ones, while expression patterns of different genes increased or decreased during plant development. Some genes displayed higher transcript levels during plant development consistent with accumulation of carotenoids during plant development. The transcript levels of *PSY1*, *LCY- β1*, *LCY-β2* and *GGPPS* were positively associated with β-carotene and lutein accumulation in all cultivars at seedling size (Fig. 5). For those four genes, the transcript expression levels were highest in 'Dragoon', 'Parris Island', 'Salinas 88' and 'Dark Land', while in 'Grand Rapids' and 'Verônica' the same genes were down regulated at the seedling sampling stage.

In the sampling at 40 DAS a similar pattern was observed for these genes, whose transcript levels (excepting *LCY-β2)* remained positively associated with the β-carotene and lutein concentration. In the inner leaves, the transcript levels of *PSY1*, *LCY- β1* and *GGPPS* was highest in the dark cultivars Parris Island and Dark Land. The light green cultivars Verônica and Grand Rapids had the lowest transcript levels for these three genes in most cases. The cultivars Dragoon and Salinas 88, that displayed a decrease of carotenoid concentration in inner leaves (probably due the plant architecture), displayed a lower level of *GGPPS* and *PSY1* transcripts, respectively (Fig. 5). For the outer leaves at 40 DAS, only *PSY1* and *LCY-β1* had expression levels that were positively correlated with carotenoid accumulation.

'Grand Rapids' and 'Verônica' had the lowest transcript levels of both genes, unlike the dark cultivars (Fig. 6).

At commercial stage size, transcript levels were highest in inner leaves than in outer ones. With the exception of *DXR*, *CHY*, *VDE* and *ZEP*, the transcript levels of carotenoid biosynthesis genes in inner leaves decreased substantially from 40 DAS to commercial size (Fig. 5). In outer leaves, some of the carotenoid biosynthesis genes displayed a minor increase in transcript levels (e.g. *GGPPS*, *PSY1*, *ZDS*, *LCY- β1*, *LCY- β2* and *CHY* whose levels increased from 40 DAS to commercial size) (Fig. 6). At the commercial stage size, carotenoid accumulation was related with different genes from those related during previous developmental stages. CRTISO had a positive relation with inner commercial leaves and was negative related with outer ones. Likewise, LCY-β2 transcripts followed the same direction of carotenoid accumulation in inner commercial leaves and VDE was related with carotenoid accumulation in outer ones (Fig. 2, Supplementary Fig. S1 and Fig. S2).

3.5 Correlations between transcript levels of carotenoid biosynthesis genes and carotenoid concentration in lettuce leaves

To identify carotenoid biosynthesis genes whose expression levels correlate with carotenoid concentrations in commercial size stage lettuce leaves, a matrix of Pearson correlation analysis was conducted between the concentrations of β-carotene, lutein and total chlorophylls in plants at commercial size and the relative gene expression at each developmental stage (Supplementary Table S4).

Significant correlations were found between *GGPPS*, *PSY1* and *LCY-β1* at seedling size and final concentrations of total chlorophyll, lutein and β-carotene in outer leaves at commercial size. *GGPPS* did not show a significant correlation with final concentration of carotenoids, but displayed a very

significant correlation (data not shown) with total chlorophyll ($P < 0.01$), which was directly correlated with β-carotene levels at seedling sampling. This could suggest that *GGPPS* is indirectly related with β-carotene synthesis at the early seedling stage. The expression of *PSY1* at seedling size displayed significant correlation with chlorophyll ($P < 0.01$), lutein and β-carotene ($P < 0.05$) in outer commercial leaves, while in inner leaves at 40 DAS correlation coefficients of 0.82 ($P < 0.05$), 0.85 ($P < 0.05$) and 0.89 ($P < 0.01$) were observed for total chlorophyll, lutein and β-carotene, respectively, when related to final concentrations in inner commercial leaves. Significant correlations were also identified for LCY-β1 transcript levels. The expression of LCY-β1 at seedling size showed correlation coefficients of 0.98 ($P < 0.001$) to final concentrations of chlorophyll and 0.77 ($P < 0.05$) to β-carotene in outer leaves. A positive correlation to chlorophyll and β-carotene levels was found for LCY-β1 expression in inner leaves at 40 DAS developmental stage. In outer leaves at 40 DAS a significant correlation between IPI transcript levels and total chlorophyll was found ($r = 0.92$, $P > 0.01$).

In the commercial size samples, there was a correlation detected for *DXS*, *PSY1*, *CRTISO*, *LCY-β1*, *LCY-β2* and *VDE* with total chlorophyll, lutein and/or β-carotene levels. *DXS* transcript levels in outer leaves is negatively correlated with total chlorophyll in inner ones ($r = - 0.90$, $P > 0.01$). This negative correlation is consistent with reduced levels of expression at later stages for a gene which is involved in the first step of the biosynthetic pathway. Indeed, DXS expression levels were significantly reduced for all genotypes examined compared with other genes tested, suggesting a significant reduction in inner pigment synthesis in older tissues (Fig 6). Conversely, the *PSY1* gene in inner commercial size stage leaves is positively correlated with total chlorophyll in outer leaves ($r = 0.85$, $P > 0.05$), which suggests an indirect relation to β-carotene. *CRTISO* transcripts from inner leaves were positively correlated with

carotenoids in both the young and outer commercial size stage leaves. However, when the samples were from outer leaves, the correlation is negative, suggesting a reduction of *CRTISO* activity in old tissues. Moreover, in outer leaves the *CRTISO* gene is down-regulated, similar to the pattern observed for *DXS* (Fig. 6). For *LCY-β1 a* coefficient of 0.80 ($P > 0.05$) was found between *LCY-β1* transcript levels and β-carotene accumulation in outer leaves. *LCY-β2* expression levels in inner leaves were correlated with chlorophyll and carotenoid accumulation. The only later-stage pathway gene related with carotenoid accumulation was *VDE*, whose expression in young commercial size leaves was correlated with β-carotene accumulation in outer leaves, despite the higher expression observed even though metabolite accumulation was reduced in commercial size leaves (Fig. 4 and Fig. 5).

4 DISCUSSION

4.1 Changes in carotenoid accumulation related to lettuce genotype and developmental stage

In this study, reverse HPLC was combined with qRT-PCR analysis to characterize carotenogenesis during lettuce plant development across cultivars which display contrasting green colour intensity. The carotenoid concentration varied according to plant development stage, increasing from seedling size to 40 DAS stage, and typically reducing when plants reached the commercial size. The lettuce cultivars tested displayed a significant variation in chlorophyll, β-carotene and lutein levels.

To better understand the relationship between expression levels of carotenoid biosynthesis pathway genes and carotenoid levels, we investigated the gene expression levels underlying enzymes involved in the control of the flow and accumulation of carotenoids in lettuce plants along the plant life cycle

in inner and outer leaves. Lutein and β-carotene were identified in different age leaves for all cultivars tested. The levels of these carotenoids at commercial size varied comparing with those described for other green leaves vegetables, such as basil (Kopsell *et al.*, 2005), *Brassicaceae* (Kopsell *et al.*, 2004) and kale (Lefsrud, 2006). The levels of β-carotene in lettuce were higher than those detected in basil but the same as those detected in *Brassicaceae* and kale. Lutein levels detected in lettuce were lower than those observed in the other green leafy vegetables where studies have been published. In a previous lettuce study (Mou, 2005), three of the same genotypes of this study were analysed for levels of β-carotene and lutein. In that study, 'Dark Land' had a higher concentration of lutein and similar levels of β-carotene, while for 'Parris Island' a similar concentration of lutein and a lower concentration of β-carotene was detected. The major difference between our study and that of Mou (2005) was for the cultivar Salinas 88, which in the Mou (2005) study displayed approximately ten fold lower levels of both carotenoids in the same leaf position. These differences between the two studies could be due to our use of outer leaves, that had more green color intensity than the internal leaves used in the Mou (2005) study.

4.2 Transcript levels relationships with carotenoid accumulation levels

The light green leaf colour in the Verônica and Grand Rapids cultivars was consistent with the lower concentrations of both carotenoids analysed, while the dark green colour observed in 'Dark Land' and 'Parris Island' was consistent with higher concentrations detected. The relationship between color intensity and carotenoid levels was also evident for 'Dragoon' and 'Salinas 88', which displayed significant differences in carotenoid concentrations from outer to inner leaves. Such a correlation between levels of carotenoids and green color intensity is likely related to the protective role of these compounds in the

photosynthetic matrix, where carotenoids are essential for avoiding chlorophyll degradation (Dall'Osto *et al.*, 2010; Demmig-Adams and Adams, 2006; Li *et al.*, 2009). The link between carotenoid accumulation and chlorophyll levels is highighted by the correlation of *GGPPS* expression during early development with final chlorophyll concentration, as well as the correlation observed for *PSY1* in inner commercial leaves (Supplementaty tables S2 and S3).

PSY1, *LCY-β1*, *GGPPS*, *DXS*, *CRTISO* and *LCY-β2* transcripts were detected in all genotypes.*GGPPS* and *PSY1* are early pathway enzymes controlling the metabolite flux into carotenoid pathway. *GGPPS* is the enzyme mediator of *GGPP* synthesis, which is a substrate for synthesis of phytoene by phytoene synthase. Our study determines that GGPP levels were positively correlated with chlorophyll and carotenoid accumulation.

A similar relationship carotenoid synthesis related with overexpression of *PSY1* has been shown in *Brassica napus* and Arabidopsis (Lindgren *et al.*, 2003; Shewmaker *et al.*, 1999). *PSY1*, the first early pathway gene directly correlated with carotenoid flux is critical in lettuce carotenoid synthesis. The substrates for *LCY-β* enzyme are lycopene, which is converted to β-carotene, and δ-carotene which is converted to α-carotene. The β-cyclase (*LCY-β*) role in synthetize β-carotene would not be possible without the synthesis of previous enzymes. Lycopene beta cyclase also had been described in others studies like the critical enzyme controlling the flux of metabolites in carotenoid pathway (Arango *et al.*, 2010; Shewmaker *et al.*, 1999; Welsch *et al.*, 2010)

The reduction in expression levels of carotenoid biosynthesis genes that were highly expressed at early stages, and their possible replacement by other early pathway genes when commercial size is reached, could suggest that the synthesis of carotenoids is constant. A correlation of *CRTISO* and *LCY-β2* transcript levels with chlorophyll and carotenoids was not oberved during early stages of development, but was detected at commercial size stage. A similar

pattern was oberved in apple fruits, wherein the expression of some genes is predominant after pollination, but the expression of these genes is reduced before the ripening stage, when *LCY-ε* is more highly expressed (Ampomah-Dwamena *et al.*, 2012). The *CRTISO* differential expression between inner and outer commercial size stage leaves suggests that this gene could be associated with a pause in carotenoid synthesis. In inner leaves *CRTISO* was more highly expressed while the carotenoid synthesis was found to be constant, following the same pattern of accumulation between cultivars (Fig. 2 and Supplementary Fig. S1). In contrast, in outer commercial leaves the *CRTISO* expression was different, with higher transcript levels detected in dark green cultivars. This observation was confirmed by the positive and negative correlations in inner and outer leaves and can help to explain the lower carotenoid levels detected in the end of the cycle (Fig. 4, Supplementaries Fig. S1, S2 and Tab. S3).

The difference in carotenoid accumulation between lettuce cultivars could also be explained by degradative enzyme action. Some early pathway genes displayed a reduction in total transcript levels at commercial size stage (Fig. 5). An increase of total transcript levels of *VDE* observed when cultivars reduced their carotenoid concentration, could be suggestive of degradation of synthesized carotenoids in internal leaves that would reduce the final carotenoid concentration. Our results indicate a reduction in β-carotene levels in inner leaves of 'Salinas 88' and 'Dragoon' when they reached the commercial size. The production of carotenoids after synthesis of phytoene in these cultivars may be influenced by the action of cleavage enzymes during carotenoid synthesis. A similar mechanism is suggested for crysanthemum, in which white cultivars had the same intermediate enzymes of a yellow cultivar, with differences in expression levels of carotenoid cleavage genes (*CCDs*), that exist only in white petal cultivars (Kishimoto and Ohmiya, 2006). Other studies have demonstrated that the pool of carotenoids could be partly determined by the rate of carotenoid

cleavage dioxygenases, depending the substrate type (Auldridge *et al.*, 2006; Garcia-Limones *et al.*, 2008; Kato *et al.*, 2006; Ohmiya *et al.*, 2006).

A range of studies have indicated that the *LCY-ε* transcript level can be directedly related to increased levels of lutein (Ampomah-Dwamena *et al.*, 2012; Clotault *et al.*, 2008; Kishimoto and Ohmiya, 2006). However, in our study *LCY-ε* expression showed no significant correlation with lutein content in lettuce (Supplementary Table S4). Nonetheless, there was a co-expression between *LCY-ε* and early pathway genes along cycle. Co-expression was observed with *LCY-β1* at seedling size and with *DXR* at 40 DAS and at commercial size stage. Co-expression between *LCY-ε* and *VDE* was also detected at 40 DAS in outer and inner leaves, as well as with *ZEP* at commercial size (data not shown). This could suggest that the synthesis of lutein is predominantly derived from expression of early pathway genes and a degradation process occurs along plant development and at the end of the pathway. Nevertheless, unlike β-carotene the level of lutein did not vary across plant development suggesting that the synthesis and degradation of lutein may occur at a constant rate along lettuce cycle. Furthermore, the expression of the late pathway genes *VDE* and *ZEP* was higher at seedling and commercial size plants, when the carotenoid level was lower (Fig. 5 and Fig. 6). The same genes displayed reducted expression levels at 40 DAS, when the carotenoid content was maximum in all lettuce cultivars. At the commercial size stage, *VDE* had a decreased level of transcripts for the cultivar Dark Land, while *ZEP*, despite the absence of correlation, displayed a transcript reduction in 'Salinas 88' and 'Parris Island'. Both of these cultivars displayed the highest concentration of both carotenoids in outer leaves at commercial size (Fig. 3 and Fig. 6).

By investigating gene expression levels combined with carotenoid levels across contrasting color (and architecture) lettuce genotypes, this study reveals the relationship between gene expression and carotenoid metabolite

accumulation in different age leaves at different times of lettuce cycle. Carotenoid accumulation was coordinated with increasing and decreasing in transcripts level of target genes, indicating that the synthesis and degradation of carotenoids in lettuce is likely regulated by expression levels of some key genes in the carotenoid biosynthesisi pathway. Contrary of fruit species, in which the accumulation of carotenoids takes place in chromoplasts and have a role to attract dispersal organisms, in photosynthetic tissues, carotenoid accumulation is in the chloroplasts and act as photosynthetic accessories (Li *et al.*, 2009). The variation in carotenoid accumulation across the lettuce life cycle could also been affected by source-sink relationships. Because in green tissues chloroplasts are not converted to chromoplasts, carotenoids are arranged in to crystals and the accumulation capacity is raised (Maass *et al.*, 2009b). If this is happening during lettuce development, it could help explain why β-carotene levels increase during development, but then rapidly decrease in the last 20 days of cycle.

Our study highlights that carotenoids and chlorophyll accumulation levels vary along development and leaf age of lettuce plants. Our results suggest that the expression levels some of the key genes along carotenoid biosynthetic pathway could be causal for these differences. Our correlation analyses between gene expression and carotenoid content in lettuce has identified the genes that can be used as candidate biomarkers at seedling stage to predict β-carotene and lutein levels at the commercial size stage. Our study provides a basis for further investigations to elucidate and apply the mechanisms underlying carotenoid biosynthesis in lettuce so that nutritionally improved lettuce varieties can be developed.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Transcript levels of lettuce carotenoid biosynthesis in inner young leaves of commercial size lettuce cultivars. plants. Gene expression levels were measured relative to the lettuce *ubiquitim* gene. Error bars represent standard deviation of the mean from three biological replicates.

Supplementary Fig. S2. Transcript levels of lettuce carotenoid biosynthesis in outer mature leaves of commercial size lettuce cultivars plants. Gene expression levels were measured relative to the lettuce *ubiquitim* gene. Error bars represent standard deviation of the mean from three biological replicates.

Supplementary Table S1. Primer sequences (designed by Quantprime) used for qRT-PCR measurements.

Supplementary Table S2. β-carotene, lutein and chlorophyll concentrations ($\mu g.100g^{-1}$ FW) in inner leaves of lettuce measured at seedling, 40 days after sowing (DAS) and at commercial size stages.

Supplementary Table S3. β-carotene, lutein and chlorophyll concentrations ($\mu g.100g^{-1}$ FW) in outer leaves of lettuce measured at seedling, 40 days after sowing (DAS) and at commercial size stages.

Supplementary Table S4. Pearson's correlation (r) comparing relative gene expression with total chlorophyll, lutein and β-carotene in inner and outer lettuce leaves at seedling stage, 40 days after sowing (DAS) stage and commercial size stage.

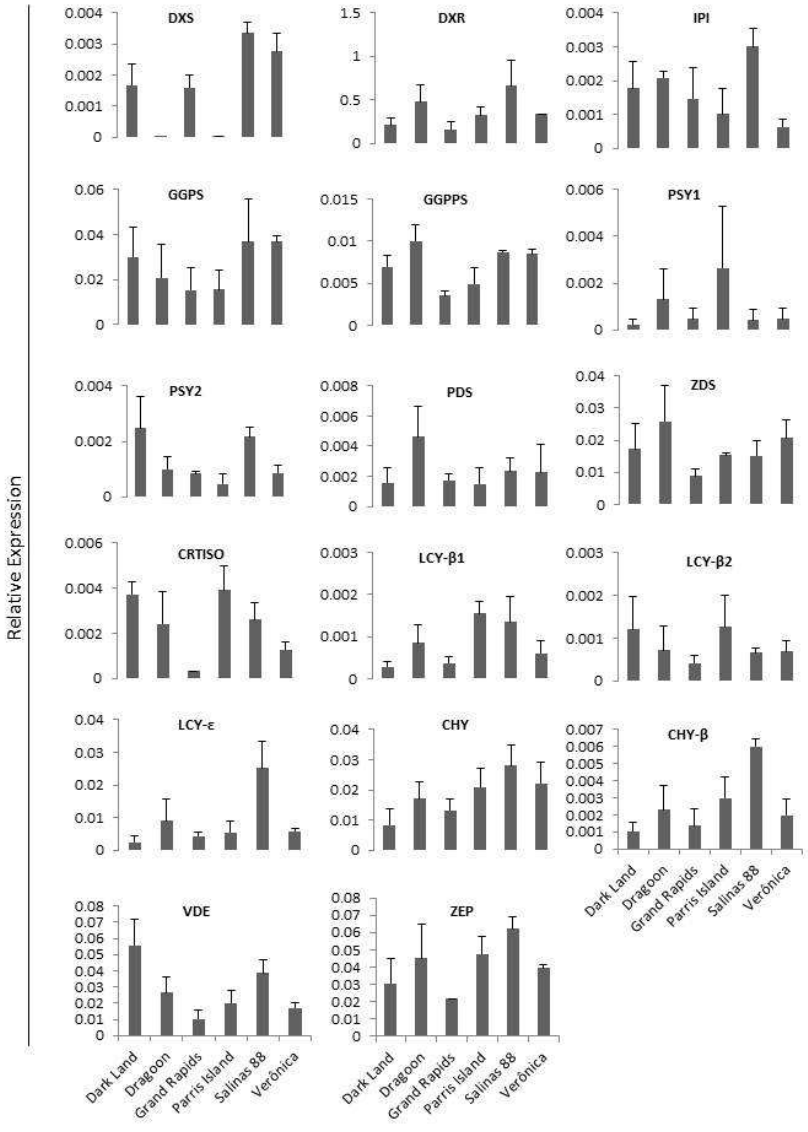


Fig. S1. Transcript levels of lettuce carotenoid biosynthesis in inner young leaves of commercial size lettuce cultivars plants. Gene expression levels were measured relative to the lettuce *ubiquitim* gene and the error bars represent standard deviation of the mean from three biological replicates.

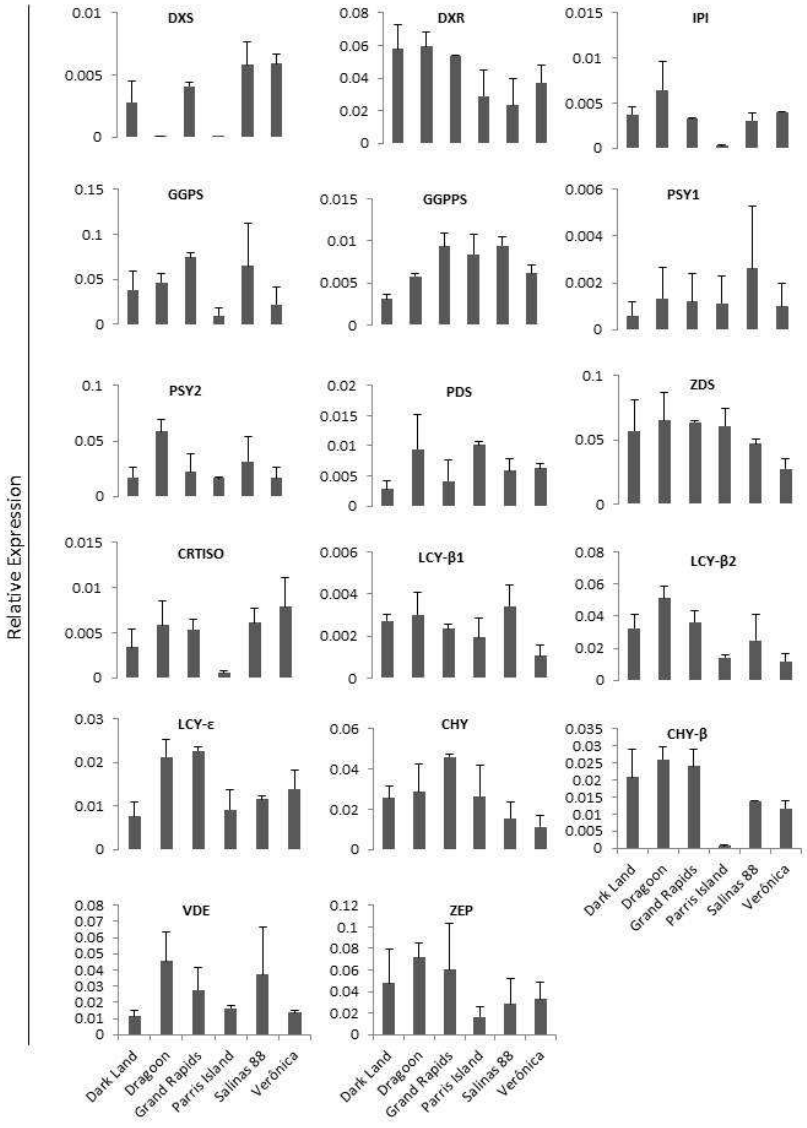


Fig. S2. Transcript levels of lettuce carotenoid biosynthesis in outer mature leaves of commercial size lettuce cultivars plants. Gene expression levels were measured relative to the lettuce *ubiquitim* gene and the error bars represent standard deviation of the mean from three biological replicates.

Table S1. Primer sequences (designed by Quantprime) used for qRT-PCR measurements.

Locus	Foward primer	Reverse primer	Accession number
UBQ	5'-CCATTTGGTCCTCCGTCTCC-3'	5'-CGAGGGTTCTGCCGTCTTCT-3'	DW141651
DXS	5'-CGCCATTGATGACAGACCCAG-3'	5'-GCCCTTCCAGCATTATTCGC-3'	AB205044
DXR	5'-AGAAACGAATCTTTGGTTGAAG-3'	5'-TCACACAATCAGGATGACGG-3'	AB205045
IPI	5'-TCACTTGACGGAGAAGATTGAG-3'	5'-CCATACCAAAGGAAATGTCACC-3'	AB205048
GGPS	5'-AATTCGGTAAATCAAGCCCTAG-3'	5'-ATACACAGCATCGGTCGGAC-3'	AB205047
GGPPS	5'-AACGCCACCCACAATCCATGAAG-3'	5'-ATGCAGAGAATGGGCCTCACAC-3'	DY975567
PSY1	5'-ACGACATCGTACACCATCTGCTC-3'	5'-TTCCAGGGTTGTGGTGGCTAAC-3'	DY974614
PSY2	5'-GCTTGACGCTGCTTTATCAGAC-3'	5'-TCTTCAGATCCATCCTCATTCC-3'	AB205050
PDS	5'-TAGCTGATGCAGGTCACAAGCC-3'	5'-TTCCAAGCTGCCACCTTTCCAC-3'	DY972145
ZDS	5'-ATCCACCTCATGCCCTTGATCC-3'	5'-TATCATAGGTGCTGGCCTTGCTG-3'	DY960874
CRTISO	5'-ATCTGTGATGTTGCGATTCAGC-3'	5'-ACGGTGGTTGGATCGGGTATC-3'	AB205043
LCY-β1	5'-AGGAAGATCAGGGCCAATGAGTCC-3'	5'-TCTTGCTGCAGAGTCAGCCAAAC-3'	DY968060
LCY-β2	5'-AAGGGTCTCGTGGTGGATC-3'	5'-TGAGGTGAAGGGTCAATCG-3'	AB205041
LCY-ε	5'-TCACACATCGTGCTAATGGATC-3'	5'-CCGTTGAAGATAAAGAAGACCC-3'	AB205046
CHY	5'-TAACCGGAAGGAGCGTGAACTG-3'	5'-TTTGCCTGCACTCCTCTTGGAC-3'	DY970341
CHYβ	5'-TCCGAACGCTTTACTTACCTTG-3'	5'-GAACATCTCCACCCTCCATTTG-3'	AB205042
VDE	5'-ACTCGCAACAATCGTCCTGAC-3'	5'-GGGCACACATTTCTTTCGG-3'	AB205051
ZEP	5'-GTTGGGTATCGGGTGTTCTTGG-3'	5'-GGCTGGTTCGTTGTGAAATGC-3'	AB205053

Table S2. β-carotene, lutein and chlorophyll concentrations (μg. g^{-1} FW) in inner leaves of lettuce measured at seedling, 40 days after sowing (DAS) and at commercial size stages.

	Dark Land			**Dragoon**			**Grand Rapids**		
Compound	**Sdl[1]**	**40 DAS**	**Comm[2]**	**Sdl**	**40 DAS**	**Comm**	**Sdl**	**40 DAS**	**Comm**
Lutein	19.5	39.7	33.8	17.1	29.7	26.5	14.3	25.9	27.4
β-carotene	65.2	288.6	110.4	55.7	192.5	72.0	42.7	102.8	71.9
Chl *a*	1117.5	1089.6	653.2	1111.0	1024.0	666.9	617.0	223.7	402.9
Chl *b*	581.9	693.9	448.8	556.5	665.6	388.3	325.5	142.7	287.2
Chl Total	1699.3	1783.5	1102.0	1667.5	1689.6	1055.2	942.5	366.3	690.2
	Parris Island			**Salinas 88**			**Verônica**		
Compound	**Sdl**	**40 DAS**	**Comm**	**Sdl**	**40 DAS**	**Comm**	**Sdl**	**40 DAS**	**Comm**
Lutein	16.7	31.0	35.0	17.9	29.8	29.7	14.5	24.2	24.8
β-carotene	52.2	129.6	102.0	63.5	94.0	70.9	42.5	59.1	64.9
Chl *a*	1135.7	1275.6	1144.6	1312.6	622.2	307.6	537.1	606.9	255.0
Chl *b*	647.2	827.3	587.0	589.3	331.8	135.7	251.0	991.7	116.6
Chl Total	1782.9	2102.9	1731.6	1901.9	1196.0	443.3	788.1	1598.6	371.6

[1]Seedling size; [2] Commercial size

Table S3. β-carotene, lutein and chlorophyll concentrations (μg. g^{-1} FW) in outer leaves of lettuce measured at seedling, 40 days after sowing (DAS) and at commercial size stages.

	Dark Land		**Dragoon**		**Grand Rapids**	
Compound	**40 DAS**	**Comm[1]**	**40 DAS**	**Comm**	**40 DAS**	**Comm**
Lutein	26.0	31.0	25.1	27.3	16.4	27.3
β-carotene	197.0	115.1	164.8	98.1	75.9	62.3
Chl *a*	1127.5	1211.5	1127.4	1470.8	503.8	346.6
Chl *b*	661.0	575.1	770.0	743.1	400.2	152.9
Chl Total	1788.5	1786.5	1897.4	2213.9	904.1	499.5
	Parris Island		**Salinas 88**		**Verônica**	
Compound	**40 DAS**	**Comm**	**40 DAS**	**Comm**	**40 DAS**	**Comm**
Lutein	22.3	30.6	23.4	30.1	15.5	23.8
β-carotene	147.1	89.3	153.5	111.6	53.7	54.9
Chl *a*	1143.4	1217.2	1168.0	1304.8	984.6	596.5
Chl *b*	741.2	646.3	667.9	485.1	1182.2	353.4
Chl Total	1884.6	1863.5	1706.2	1911.2	2166.8	949.8

[1] Commercial size

Table S4. Pearson's correlation (r) comparing relative gene expression with final total chlorophyll, lutein and β-carotene in inner and outer lettuce leaves at seedling stage, 40 days after sowing (DAS) stage and commercial size stage.

		Seedling *vs* commercial		
Gene	**Prevision**	**Chl T**	**Lutein**	**β-carotene**
DXS	inner	0.56	0.64	0.41
	outer	0.21	0.43	0.07
DXR	inner	-0.17	-0.40	-0.55
	outer	0.23	-0.02	0.15
IPI	inner	0.63	0.02	0.17
	outer	-0.11	-0.20	-0.40
GGPS	inner	-0.33	-0.36	-0.33
	outer	-0.90	-0.36	-0.67
GGPPS	inner	0.53	0.43	0.25
	outer	0.92**	0.59	0.72
PSY	inner	0.71	0.72	0.65
	outer	0.92**	0.78*	0.84*
PSY2	inner	0.50	0.34	0.34
	outer	-0.42	0.23	-0.27
PDS	inner	-0.41	-0.72	-0.77
	outer	0.15	-0.40	-0.04
ZDS	inner	-0.10	-0.46	-0.47
	outer	0.38	-0.13	0.22
CRTISO	inner	0.47	0.39	0.46
	outer	-0.13	0.45	0.14
LBC	inner	0.42	0.39	0.26
	outer	0.98***	0.53	0.77*
LCY-β	inner	0.73	0.70	0.65
	outer	-0.16	0.52	-0.03
LCY-ε	inner	0.72	0.54	0.39
	outer	-0.05	0.42	-0.09
CHY	inner	-0.10	-0.32	-0.37
	outer	0.66	-0.20	0.26
CHY-β	inner	0.68	0.23	0.08
	outer	0.22	0.16	-0.10

Continuation…				
VDE	inner	0.51	0.11	0.17
	outer	-0.20	0.11	-0.19
ZEP	inner	0.56	0.42	0.58
	outer	0.42	0.55	0.57

		40 DAS inner *vs* commercial		
Gene	**Prevision**	**Chl T**	**Lutein**	**β-carotene**
DXS	inner	0.11	0.69	0.50
	outer	-0.10	0.66	0.29
DXR	inner	-0.39	-0.68	-0.83
	outer	-0.06	-0.40	-0.22
IPI	inner	-0.04	-0.64	-0.50
	outer	-0.04	-0.54	-0.33
GGPS	inner	-0.48	-0.87	-0.73
	outer	-0.67	-0.90	-0.83
GGPPS	inner	0.37	0.73	0.69
	outer	0.71	0.70	0.79*
PSY	inner	0.82*	0.85*	0.89**
	outer	0.74	0.77	0.73
PSY2	inner	0.66	0.16	0.37
	outer	0.15	0.10	0.03
PDS	inner	-0.11	-0.54	-0.19
	outer	-0.03	-0.59	-0.24
ZDS	inner	-0.52	-0.32	-0.40
	outer	-0.43	-0.52	-0.54
CRTISO	inner	-0.32	-0.41	-0.56
	outer	-0.75	-0.38	-0.70
LBC	inner	0.32	0.26	0.13
	outer	0.96***	0.46	0.76*
LCY-β	inner	-0.42	-0.44	-0.72
	outer	-0.25	-0.41	-0.46
LCY-ε	inner	0.40	0.01	0.19
	outer	-0.08	0.08	-0.02
CHY	inner	0.23	-0.12	0.21
	outer	-0.09	-0.45	-0.36

Continuation…

CHY-β	inner	0.31	0.01	-0.16
	outer	0.75	0.18	0.39
VDE	inner	0.27	-0.27	-0.09
	outer	0.05	-0.15	-0.07
ZEP	inner	0.54	0.12	0.19
	outer	0.70	0.27	0.49

		40 DAS outer *vs* commercial		
Gene	**Prevision**	**Chl T**	**Lutein**	**β-carotene**
DXS	inner	-0.61	0.01	-0.20
	outer	-0.23	0.12	0.09
DXR	inner	-0.67	-0.39	-0.66
	outer	-0.30	-0.10	-0.10
IPI	inner	0.92**	0.65	0.55
	outer	0.54	0.61	0.36
GGPS	inner	-0.48	-0.91	-0.78
	outer	-0.14	-0.77	-0.40
GGPPS	inner	-0.46	-0.18	-0.46
	outer	0.25	0.22	0.40
PSY	inner	0.07	-0.08	-0.20
	outer	0.61	0.30	0.57
PSY2	inner	-0.66	-0.66	-0.76
	outer	0.23	-0.43	0.04
PDS	inner	-0.13	-0.47	-0.50
	outer	0.22	-0.12	0.12
ZDS	inner	0.00	-0.35	-0.43
	outer	0.28	-0.01	0.16
CRTISO	inner	-0.63	-0.81	-0.88
	outer	-0.15	-0.48	-0.21
LBC	inner	-0.16	0.04	-0.24
	outer	0.39	0.45	0.54
LCY-β	inner	-0.58	-0.80	-0.93
	outer	-0.39	-0.76	-0.66
LCY-ε	inner	-0.67	-0.45	-0.69
	outer	-0.40	-0.16	-0.20

Continuation…				
CHY	inner	-0.15	-0.37	-0.62
	outer	0.01	0.45	0.30
CHY-β	inner	-0.75	-0.71	-0.78
	outer	-0.27	-0.72	-0.47
VDE	inner	-0.79	-0.57	-0.81
	outer	-0.22	-0.36	-0.21
ZEP	inner	0.09	0.28	0.00
	outer	0.66	0.63	0.74

		Commercial inner vs commercial		
Gene	**Prevision**	**Chl T**	**Lutein**	**β-carotene**
DXS	inner	-0.85	-0.31	-0.40
	outer	-0.35	-0.21	-0.08
DXR	inner	-0.26	-0.16	-0.40
	outer	0.60	0.11	0.45
IPI	inner	-0.23	0.06	-0.12
	outer	0.51	0.49	0.73
GGPS	inner	-0.67	-0.25	-0.25
	outer	0.09	-0.19	0.17
GGPPS	inner	-0.34	-0.40	-0.35
	outer	0.57	-0.24	0.34
PSY	inner	0.37	0.43	0.18
	outer	0.85*	0.54	0.65
PSY2	inner	-0.28	0.27	0.29
	outer	0.33	0.49	0.73
PDS	inner	-0.11	-0.55	-0.49
	outer	0.45	-0.34	0.12
ZDS	inner	0.05	-0.30	-0.14
	outer	0.59	-0.30	0.19
CRTISO	inner	0.70	0.82*	0.79*
	outer	0.81*	0.76*	0.76*
LBC	inner	0.36	0.35	0.03
	outer	0.53	0.36	0.27
LCY-β	inner	0.77*	0.85*	0.91**
	outer	0.55	0.63	0.50

Continuation…

LCY-ε	inner	-0.42	-0.12	-0.42
	outer	0.37	0.20	0.41
CHY	inner	-0.31	-0.21	-0.51
	outer	0.18	-0.14	-0.05
CHY-β	inner	-0.22	0.07	-0.28
	outer	0.41	0.29	0.39
VDE	inner	0.08	0.49	0.56
	outer	0.59	0.63	0.86*
ZEP	inner	-0.02	0.11	-0.16
	outer	0.67	0.25	0.46

		Commercial outer vs commercial		
Gene	**Prevision**	**Chl T**	**Lutein**	**β-carotene**
DXS	inner	-0.90	-0.44	-0.51
	outer	-0.55	-0.37	-0.29
DXR	inner	0.10	-0.19	0.13
	outer	-0.09	-0.14	0.00
IPI	inner	-0.43	-0.68	-0.47
	outer	0.12	-0.46	0.05
GGPS	inner	-0.54	-0.35	-0.44
	outer	-0.27	0.03	0.09
GGPPS	inner	-0.15	-0.11	-0.45
	outer	-0.29	0.00	-0.29
PSY	inner	0.66	0.70	0.85
	outer	0.54	0.72	0.71
PSY2	inner	-0.04	-0.40	-0.40
	outer	0.52	-0.10	0.30
PDS	inner	0.50	0.02	-0.10
	outer	0.49	-0.05	-0.03
ZDS	inner	0.64	0.41	0.40
	outer	0.29	0.56	0.38
CRTISO	inner	-0.93	-0.92	-0.86
	outer	-0.35	-0.76	-0.37
LBC	inner	0.01	0.21	0.08
	outer	0.56	0.62	0.80

Continuation…				
LCY-β	inner	0.03	-0.23	-0.11
	outer	0.24	0.08	0.31
LCY-ε	inner	-0.31	-0.73	-0.69
	outer	-0.39	-0.56	-0.49
CHY	inner	0.29	0.08	0.12
	outer	-0.36	0.15	-0.17
CHY-β	inner	-0.36	-0.47	-0.28
	outer	-0.12	-0.20	0.08
VDE	inner	-0.20	-0.41	-0.55
	outer	0.35	-0.03	0.25
ZEP	inner	-0.16	-0.48	-0.26
	outer	-0.08	-0.26	-0.03

1 Commercial size; * Significant (P < 0.05); ** Significant (P < 0.01); *** Significant (P < 0.001).

FUNDING

This work was supported by Coordenação de Pessoal de Nível Superior of Brazil Ministry of Education (CAPES – BRAZIL) [BEX 7368/13-5 to C.L.O.) and the Science Foundation Ireland (SFI) International Strategic Cooperation Award (ISCA) Programme supporting the Research Brazil Ireland Initiative (http://rbi.ie/).

ACKNOWLEDGEMENTS

The authors wish to thank the Coordenação de Pessoal de Nível Superior of Brazil Ministry of Education (CAPES – BRAZIL) and Science Fundation Ireland (SFI) that provided the support necessary for this study. We are grateful to Gerard Fahy from the Chemistry Deparment at the National University of Ireland Galway for technical support.

REFERENCES

Adefegha SA, Oboh G. 2013. Cooking enhances the antioxidant properties of some tropical green leafy vegetables. *African Journal of Biotechnology* **10**, 632-639.

Águila Ruiz-Solaa M, Rodríguez-Concepcióna M. 2012. Carotenoid Biosynthesis in Arabidopsis: A Colorful Pathway. *The Arabdopsis Book* **10**, 28.

Ampomah-Dwamena C, Dejnoprat S, Lewis D, Sutherland P, Volz RK, Allan AC. 2012. Metabolic and gene expression analysis of apple (Malus x domestica) carotenogenesis. *J Exp Bot* **63**, 4497-4511.

Ampomah-Dwamena C, McGhie T, Wibisono R, Montefiori M, Hellens RP, Allan AC. 2009. The kiwifruit lycopene beta-cyclase plays a significant role in carotenoid accumulation in fruit. *J Exp Bot* **60**, 3765-3779.

Arango J, Wust F, Beyer P, Welsch R. 2010. Characterization of phytoene synthases from cassava and their involvement in abiotic stress-mediated responses. *Planta* **232**, 1251-1262.

Auldridge ME, Block A, Vogel JT, Dabney-Smith C, Mila I, Bouzayen M, Magallanes-Lundback M, DellaPenna D, McCarty DR, Klee HJ. 2006. Characterization of three members of the Arabidopsis carotenoid cleavage dioxygenase family demonstrates the divergent roles of this multifunctional enzyme family. *Plant J* **45**, 982-993.

Cazzonelli CI, Pogson BJ. 2010. Source to sink: regulation of carotenoid biosynthesis in plants. *Trends Plant Sci* **15**, 266-274.

Cazzonelli CI, Yin K, Pogson BJ. 2009. Potential implications for epigenetic regulation of carotenoid biosynthesis during root and shoot development. *Plant Signal Behav* **4**, 339-341.

Clotault J, Peltier D, Berruyer R, Thomas M, Briard M, Geoffriau E. 2008. Expression of carotenoid biosynthesis genes during carrot root development. *J Exp Bot* **59**, 3563-3573.

Dall'Osto L, Cazzaniga S, Havaux M, Bassi R. 2010. Enhanced photoprotection by protein-bound vs free xanthophyll pools: a comparative analysis of chlorophyll b and xanthophyll biosynthesis mutants. *Mol Plant* **3**, 576-593.

de Pee S, West CE, Muhilal, Karyadi D, Hautvast JG. 1995. Lack of improvement in vitamin A status with increased consumption of dark-green leafy vegetables. *Lancet* **346**, 75-81.

Demmig-Adams B, Adams WW, 3rd. 2006. Photoprotection in an ecological context: the remarkable complexity of thermal energy dissipation. *New Phytol* **172**, 11-21.

Fraser PD, Bramley PM. 2004. The biosynthesis and nutritional uses of carotenoids. *Prog Lipid Res* **43**, 228-265.

Garcia-Limones C, Schnabele K, Blanco-Portales R, Luz Bellido M, Caballero JL, Schwab W, Munoz-Blanco J. 2008. Functional characterization of FaCCD1: a carotenoid cleavage dioxygenase from strawberry involved in lutein degradation during fruit ripening. *J Agric Food Chem* **56**, 9277-9285.

Giuliano G, Bartley GE, Scolnik PA. 1993. Regulation of carotenoid biosynthesis during tomato development. *Plant Cell* **5**, 379-387.

Hayashi E, You Y, Lewis R, Calderon MC, Wan G, Still DW. 2012. Mapping QTL, epistasis and genotype × environment interaction of antioxidant activity, chlorophyll content and head formation in domesticated lettuce (Lactuca sativa). *Theor Appl Genet* **124**, 1487-1502.

Kato M, Matsumoto H, Ikoma Y, Okuda H, Yano M. 2006. The role of carotenoid cleavage dioxygenases in the regulation of carotenoid profiles during maturation in citrus fruit. *J Exp Bot* **57**, 2153-2164.

Khachik F, Bernstein PS, Garland DL. 1997. Identification of lutein and zeaxanthin oxidation products in human and monkey retinas. *Invest Ophthalmol Vis Sci* **38**, 1802-1811.

Kishimoto S, Ohmiya A. 2006. Regulation of carotenoid biosynthesis in petals and leavesof chrysanthemum (Chrysanthemum morifolium). *Physiologia Plantarum* **128**, 436–447.

Kopsell DA, Kopsell DA, Curran-Celentano J. 2005. Carotenoids and Chlorophyll Pigments in Sweet Basil Grown in the Field and Greenhouse. *HortScience* **40**, 1230-1233.

Kopsell DA, Kopsell DE, Lefsrud MG, Curran-Celentano J, Dukach E. 2004. Variation of Lutein, β-Carotene, and Chlorophyll concentrations among Brassica oleraceae cultigens and seasons. *HortScience* **39**, 361-364.

Li Z, Wakao S, Fischer BB, Niyogi KK. 2009. Sensing and responding to excess light. *Annu Rev Plant Biol* **60**, 239-260.

Lindgren LO, Stalberg KG, Hoglund AS. 2003. Seed-specific overexpression of an endogenous Arabidopsis phytoene synthase gene results in delayed germination and increased levels of carotenoids, chlorophyll, and abscisic acid. *Plant Physiol* **132**, 779-785.

Llorach R, Martínez-Sánchez A, Tomás-Barberán FA, Gil MI, Ferreres F. 2008. Characterisation of polyphenols and antioxidant properties of five lettuce varieties and escarole *Food Chemistry* **108**, 1028-1038.

Lu S, Van Eck J, Zhou X, Lopez AB, O'Halloran DM, Cosman KM, Conlin BJ, Paolillo DJ, Garvin DF, Vrebalov J, Kochian LV, Küpper H, Earle ED, Cao J, Li L. 2006. The cauliflower Or gene encodes a DnaJ cysteine-rich domain-containing protein that mediates high levels of beta-carotene accumulation. *Plant Cell* **18**, 3594-3605.

Maass D, Arango J, Wüst F, Beyer P, Welsch R. 2009a. Carotenoid crystal formation in Arabidopsis and carrot roots caused by increased phytoene synthase protein levels. *PLoS One* **4**, e6373.

Maass D, Arango J, Wüst F, Beyer P, Welsch R. 2009b. Carotenoid crystal formation in Arabidopsis and carrot roots caused by increased phytoene synthase protein levels. *PLoS One* **4**, e6373.

Mou B. 2005. Genetic Variation of Beta-carotene and Lutein Contents in Lettuce. *Journal of the American Society for Horticultural Science* **130**, 7.

Nakkanong K, Yang JH, Zhang MF. 2012. Carotenoid accumulation and carotenogenic gene expression during fruit development in novel interspecific inbred squash lines and their parents. *J Agric Food Chem* **60**, 5936-5944.

Norris SR, Barrette TR, DellaPenna D. 1995. Genetic dissection of carotenoid synthesis in arabidopsis defines plastoquinone as an essential component of phytoene desaturation. *Plant Cell* **7**, 2139-2149.

Ohmiya A, Kishimoto S, Aida R, Yoshioka S, Sumitomo K. 2006. Carotenoid cleavage dioxygenase (CmCCD4a) contributes to white color formation in chrysanthemum petals. *Plant Physiol* **142**, 1193-1201.

Raju M, Varakumar S, Lakshminarayana R, Krishnakantha TP, Baskaran V. 2007. Carotenoid composition and vitamin A activity of medicinally important green leafy vegetables. . *Food Chemistry* **101**, 1598-1605.

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

Tuan PA, Park NI, Park WT, Kim YB, Kim, Kwang J, Lee J, Lee S-h, Yang T-J, Park SU. 2012. Carotenoids accumulation and expression of carotenogenesis genes during seedling and leaf development in Chinese cabbage (Brassica rapa subsp. pekinensis). *Plant omics* **5**, 143-148.

Vishnevetsky M, Ovadis M, Vainstein A. 1999. Carotenoid sequestration in plants: the role of carotenoid-associated proteins. *Trends Plant Sci* **4**, 232-235.

Welsch R, Arango J, Bar C, Salazar B, Al-Babili S, Beltran J, Chavarriaga P, Ceballos H, Tohme J, Beyer P. 2010. Provitamin A accumulation in cassava

(Manihot esculenta) roots driven by a single nucleotide polymorphism in a phytoene synthase gene. *Plant Cell* **22**, 3348-3356.

West KP. 2002. Extent of vitamin A deficiency among preschool children and women of reproductive age. *J Nutr* **132**, 2857S-2866S.

West KPJ, Darnton-Hill I. 2001. Vitamin A deficiency. in Nutrition and health indeveloping countries. *Humana Press*, 267-306.

Wu X, Beecher GR, Holden JM, Haytowitz DB, Gebhardt SE, Prior RL. 2004. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *J Agric Food Chem* **52**, 4026-4037.

Yeum KJ, Russell RM. 2002. Carotenoid bioavailability and bioconversion. *Annu Rev Nutr* **22**, 483-504.

ARTIGO 2

ABSORPTION OF LUTEIN AND β-CAROTENE OF LETTUCE GENOTYPES IS DIRECTED RELATED TO DISRUPTION OF FOOD MATRIX BY COOKING

Artigo redigido nas normas da revista “Food Chemistry”

ABSTRACT

The present study assessed the profiles of lutein and β-carotene in different leaves of contrasting lettuce genotypes to amount transferred to micelles from digested raw and cooked lettuce and also determined their cellular uptake. The results indicated different initial concentration of carotenoids in fresh leaves, but suggest that these differences are not related to the absorption and transport by intestine. Disruption of the food matrix by cooking lettuce resulted in carotenoid concentration losses and in reduction of carotenoid transfer to micellar fraction, however, it was compensated by raise in bioavailability comparing with fresh leaves without heat treatment. Unless the food matrix is disrupted, the absorption of carotenoids present in biofortified lettuce is in a very low rate, similar to low carotenoids cultivars. Our data suggests that the breeding programs of biofortified crops must be related to efforts to increase the bioavailability of carotenoids present in food matrix.

Keywords: Lettuce.Lutein.β-carotene.Processing.*In vitro* digestion. Caco-2 cells.

1 INTRODUCTION

Despite the high degree of structural diversity of carotenoids and the large variation of biological functions, humans cannot synthesize these compounds and must rely on dietary sources to provide sufficient levels, such fruits and vegetables, that are primary sources of carotenoids in the human diet and whose intake has been associated with numerous health benefits (Grusak & DellaPenna, 1999; Mortensen, Skibsted, & Truscott, 2001; Nagao, 2014). The importance of vegetables like source of carotenoids is linked to the recommendation of ingesting at least seven servings of fruit and vegetables per day (http://www.cnpp.usda.gov/DietaryGuidelines). However, the average consumption by adults in the USA is around the half of the recommended. In Brazil, the consumption of fruits and vegetables falls short of World Health Organization recommendation, and is reached by only 18% of population (www.ibge.gov.br).

Green leafy vegetabes have high levels of carotenoids with considerable amounts of β-carotene and lutein (Kopsell, Kopsell, & Curran-Celentano, 2005; Kopsell, Kopsell, Lefsrud, Curran-Celentano, & Dukach, 2004; Mou, 2005). Carotenoids present in these vegetables are arranged in the chloroplasts, where they act as light harvesting and protectors against photooxidative damage to chlorophylls (Águila Ruiz-Solaa & Rodríguez-Concepcióna, 2012; Cazzonelli, Yin, & Pogson, 2009). This arrangement forms the food matrix that in non-photosynthetic organs is formed in the chromoplasts. Lettuce, the most important green leaf vegetable consumed worldwide can be an important source of carotenoids, such β-carotene, the most important pro vitami A carotenoid, and lutein, the main xanthophyll carotenoid constituent of human retin. Intake of a diet rich in these two carotenoids is associated with prevention of certain cancers (Finley, 2005; Seifried, McDonald, Anderson, Greenwald, & Milner, 2003; Tang, Jin, Zeng, & Wang, 2005), cardiovascular diseases (Granado,

Olmedilla, & Blanco, 2003) and eye diseases (Johnson et al., 2000; Sommerburg, Keunen, Bird, & van Kuijk, 1998) as well as enhanced immune system functions (Garcia et al., 2003).

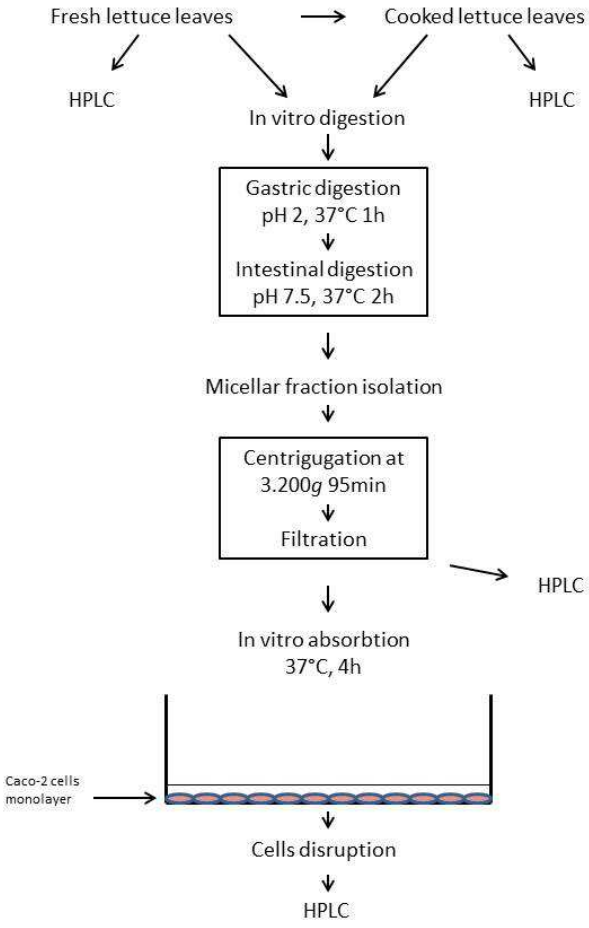
Efforts to increase the concentration of carotenoids in plants have been made by conventional plant breeding, agronomic management and genetic engineering. However, despite the high levels accumulated in vegetables, carotenoids should be released from the food matrix through disruption by processing in order to be ready for intestinal absorption in humans (Nagao, 2014). *In vitro* and *in vivo* studies show that carotenoid absorption can be influenced by the source, degree of processing, degree of isomerization during digestion, transit time in the intestine, and the nutritional status of the human subjects (Faulks & Southon, 2005).

In literature there is a range of studies that analyzed the bioaccessibility (fraction of an ingested carotenoid that is released and transferred during digestion from the food matrix to mixed micelles and thus become accessible for intestinal absorption) and bioavailability (fraction of an ingested carotenoid that is absorbed by intestine and is available for utilization in normal physiological functions and/or for storage) in various important carotenoid source vegetables (Aherne , Daly, Jiwan, O'Sullivan, & O'Brien, 2010; Courraud, Berger, Cristol, & Avallone, 2013; Ghavami, Coward, & Bluck, 2012; Netzel et al., 2010; O'Connell, Ryan, & O'Brien, 2007; O'Sullivan, Galvin, Aherne, & O'Brien, 2010; Stahl et al., 2002; Tanumihardjo, Palacios, & Pixley, 2010; Tyssandier et al., 2003; van Lieshout et al., 2001). Nonetheless, there is a lack in the literature about bioaccessibility and bioavailability of carotenoids of different content genotypes of a specific crop with contrasting carotenoid content.

In this sense, the present study examined the relation between the initial lutein and β-carotene content in fresh and cooked contrasting lettuce cultivars

and the bioaccessibility and bioavailability of these carotenoids using an *in vitro* digestion model coupled with a human intestinal Caco-2 cell model (Fig. 1).

Figure 1 Steps of the *in vitro* digestion model and Caco-2 cell culture model used in the present study.



2 MATERIALS AND METHODS

2.1 Materials

HPLC solvents, standards (β-carotene, lutein and trans-β-apo-carotenal), enzymes (porcine pepsin, porcine bile extract and pancreatin) and cell culture material (Dulbecco Modified Eagle Medium – DMEM, Fetal Bovine Serum- FBS, nonessential amino acids, L-glutamine, penicillin and streptomycin) were purchased from Sigma Aldrich Ireland (Arklow, Wicklow, Ireland). Caco-2 cells line was kindly provided by Dr. Aoife Boyd from Department of Microbiology at National University Ireland Galway. Lettuce seeds were gently provided by lettuce breeding program from Universidade Federal de Lavras, Minas Gerais, Brazil.

2.2 Plant material and growing conditions

Six commercial lettuce cultivars were chosen for analysis based on their differences in green color intensity and carotenoid concentration detected in a pilot study: Dark Land Cos MT (romaine type, dark green and high carotenoid level), Dragoon (mini romaine, dark green and high carotenoid level), Grand Rapids (batavia type, light green and low carotenoid level), Parris Island (romaine type, medium green and high carotenoid level), Salinas 88 (crisphead, medium green intensity, high carotenoid level in external leaves and low carotenoid level in internal ones) and Verônica (batavia, light green and low carotenoid level). Plants were grown at the National University of Ireland Galway, between February and March 2014, in a plant growth chamber under 16 hours day light period. Lettuce seeds were sown on soil in 200 mL pots and transplanted to three liter pots at seedling size (20 days after sowing). The experiment was conducted in a completely randomized design with three replications and three plants per plot. All plants were grown at the same time

and in the same chamber, with the same light intensity and temperature to minimize environmental variability. Leaf samples of each cultivar were harvested when commercial size was reached (60 days after sowing) and separated in outer leaves (first external leaves) and inner leaves (a leaf from the seventh inner layer of leaves from the outside of the head). In each lettuce plant, sampling was done on three different leaves around the plant in the same location in each outer or inner leaf, corresponding to a total of three pooled samples per plant, and nine pooled samples per plot. Each sample was divided in three parts to be analyzed raw, raw digested and cooked digested by HPLC.

2.3 Sample preparation and cooking procedure

The newly harvested leaves samples were manipulated under subdued light to minimize carotenoid photodegradation. The first portion on fresh leaves was prepared for immediate carotenoid extraction to determine the initial carotenoid concentration. The second part was prepared for *in vitro* digestion and the third part was boiled in individual sealed tubes with distilled water for 20 min at 98º C in a water bath (Grant Optima, model GD120, United Kindon). Cooking procedure was performed with a lettuce/water ratio of 0.14 (Courraud et al., 2013). Cooked lettuce leaves were drained with a colander during 1 min and cooled at room temperature for 5 min before further digestion or carotenoid extraction.

2.4 Sample homogenization and *in vitro* digestion protocol

The *in vitro* digestion was performed by the method used in spinach by Garret with minor modifications (Fig. 1) (Garrett, Failla, & Sarama, 2000). To prepare the lettuce meal, 50 g of lettuce leaves from each raw and cooked sample of inner and outer leaves from each lettuce cultivar were chopped finely. 35 mL of saline solution containing 150 µmol/L butylated hydroxytoluene

(BHT) were added and the samples were homogenized to a pureed consistency with a hand blender (Cookworks 967) to simulate mastication. For gastric digestion, samples (6 g) of the lettuce meal were transferred to amber bottles and diluted with 34 mL of saline (150 mmol/L) containing 150 µmol/L BHT. Briefly, the homogenized meal was acidified (pH 2) before addition of porcine pepsin to a final concentration of 1.8 mg/mL and incubated at 37°C in a shaking water bath (Thermo Scientific, model MaxQ6000, Dublin - Ireland) at 95 rpm for 1 hr. For intestinal digestion, the pH of the gastric digestate was then increased to 5.3 with sodium bicarbonate, and porcine bile extract and pancreatin were added to provide final quantities (concentrations) of 2.4 and 0.4 mg of bile extract and pancreatin per mL digestate, respectively, in a final reaction volume of 46 mL. The pH was elevated to 7.5 with 1 N sodium hydroxide and the bottles were capped and samples were incubated in the shaking water bath at 37°C for 2 hr. The digesta samples were then transferred to 50 mL tubes to micelar fraction isolation.

2.5 Isolation of the micellar fraction

The aqueous fraction was isolated from the digesta according to the method used in cassava by Thakkar *et al*. with minor modifications (Thakkar, Maziya-Dixon, Dixon, & Failla, 2007). The micellar fraction was isolated from the digesta by centrifugation of the 50 mL tubes at 3200 x *g* for 95 min at 4ºC (Eppendorf, model 5810 R). The upper phase was collect with an 18 gauge needle in a 10 mL syringe. The solution was filtered (cellulose acetate, 0.22 µm pore size; Sigma Aldrich Ireland) to remove microcrystalline non micellarized carotenoids that were not pelleted during centrifugation.

2.6 Uptake of micellar carotenoids by Caco-2 cells

Stock cultures of human colon adenocarcinoma Caco-2 cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, nonessential amino acids (1 %), L-glutamine (2 mmol/L), penicillin and streptomycin (1 %) in an incubator with humidified atmosphere of 95% air/5% CO_2 (v/v) at 37°C with medium changed every other day. Cultures of Caco-2 at passages 26-30 were grown in 24 well dishes (Sigma Aldrich Ireland) and used for experiments 12 days after reaching confluency, when the differentiation of the cells maintained in this manner is maximal (Ellwood, Chatzidakis, & Failla, 1993). Monolayers were washed two times with 1 mL Hank's balanced salts solution (HBSS) before adding 1 mL of test medium containing 0.75 mL DMEM and either 0.25 mL aqueous fraction or 0.25 mL saline (negative control) to triplicate wells for each test. Cultures were incubated in an incubator of 95% air/5% CO_2 (v/v) at 37°C for 4 hours. After incubation, the medium was removed and monolayers were washed two times with HBSS. Cells were scraped into 2 mL tubes containing 1 mL of ice-cold phosphate buffered saline containing 10% (v/v) ethanol and 45 µmol/L BHT. Tubes were tightly closed and frozen under liquid nitrogen and stored at – 80º C until analyses.

2.7 Fresh and boiled lettuce leaves, micellar fraction and cell samples extraction

Fresh leaves and boiled lettuce leaves samples were extracted immediately after harvest and boil, respectively. 0.3 g of initial tissue was ground under liquid nitrogen in a porcelain mortar and transferred to a 2 mL centrifuge tube with one glass bead. 200 µL of 80% v/v acetone was added before adding ethyl acetate (200 µL), and the tubes were agitated at 30,000 rpm for 1 min in a tissuelyser (RETSCH MM200 – Qiagen, Manchester, UK). Water (140 µL) was added and the mixture was agitated again at 30,000 rpm for 1 min

and then centrifuged at 15.800g_n for 5 min in a microcentrifuge (Heraeus Fresco 17 – Thermo Scientific, Dublin, Ireland). The carotenoid containing upper phase was then transferred to a new tube. The samples were extracted at least three more times, adding 200 µL of ethyl acetate, agitating, centrifuging at 15.800g_n for 5 min, and removing the upper phase until the leaf sediments did not have any visible green color.

Samples of micellar fraction and cells were thawed and cells were crushed in a tissuelyser (Qiagen, model RETSCH MM200 - Manchester, UK) in tubes with one glass bead for 5 min at 20,000 rpm. Previously, cells samples were examined at microscope to certificate that cells were broken after this crushing period. After cell crushing, 200 µL of 80% v/v acetone was added in cells and micellar samples before adding ethyl acetate (200 µL), and the tubes were vortexed vigorously for 1 min. Water (140 µL) was added and the mixture was vortexed again for 1 min and then centrifuged at 15.800g_n for 5 min in a microcentrifuge (Heraeus Fresco 17 – Thermo Scientific, Dublin, Ireland). The carotenoid containing upper phase was then transferred to a new tube. The samples were extracted two more times, adding 200 µL of ethyl acetate, vortexing, centrifuging at 15.800g_n for 5 min, and removing the upper phase.

2.8 Quantification of β-carotene, lutein and retinoid

The combined ethyl acetate phases of all samples were vacuum dried in a centrifugal evaporator (miVac – GeneVac SP Scientific, Ipswich, UK). The dried samples were subsequently redissolved in 1.0 mL of 0.8% of BHT/acetone (Ampomah-Dwamena et al., 2009) and analyzed by the reverse phase of high performance liquid chromatography (HPLC). The HPLC system (Alliance, Waters Co., Milford, Mass.) consisted of a separation unit (model 2695), YMC 4.6 x 10 mm C30 guard cartridge and YMC RP C30 column (3 µm, 250 × 4.6 mm - YMC, Wilmington, North Carolina, USA). The column temperature was

25 °C and samples were kept in 4 ºC sample cooler and a 50 µL aliquot was injected into a 1 mL min^{-1} flow rate. The elution was performed using a mobile phase comprising solvent A (MeOH), solvent B [H2O/MeOH, 20:80) containing 0.2% w/v ammonium acetate], and solvent C (*tert*-butyl methyl ether). The elution gradient was a time reduced version of that described by Ampomah-Dwamena et al. (2012). The gradient started with 95% A/5% B for 2 min, decreasing to 80% A/5% B/15% C between 2 and 10 min, decreasing to 30% A/5% B/65% C by 15 min, decreasing to 25% A/5% B/70% C at 20 min, and returning to 95% A/5% B at 25 min. Retinol activity equivalents (RAE) were calculated using a conversion factor of 12 for β-carotene for one retinol and expressed in mg RAE /100g (Campos & Rosado, 2005). β-Carotene and lutein were identified by comparing the retention time and absorption spectra of individual peaks with the standards. The concentrations of both β-carotene and lutein were determined using the areas under the curve ratios between compounds and internal standard trans-β-apo-carotenal. The quantification was validated using an eight point calibration curve and the results were expressed as mg per 100g and percentage of initial carotenoid concentration in fresh leaves.

2.9 Statistical analysis

Data refers to β-carotene, lutein and RAE concentrations in mg/100 g of fresh lettuce leaves, and the percentages remaining of β-carotene and lutein in cooked lettuce, micellar fraction (bioaccessibility) and in Caco-2 cells (bioavailability). The concentrations remaining in each step were expressed based on initial carotenoid concentration in fresh inner and outer leaves of each lettuce cultivar. Data are expressed as the mean ± SD of three independent experiments and were analyzed by one-way analysis of variance (ANOVA) and, where appropriate, Tukey's post hoc multiple comparison test between cultivars and age leaf in each step.

3. RESULTS AND DISCUSSION

3.1 Lutein, β-carotene, and RAE contents in fresh leaves of lettuce genotypes

Lutein, β-carotene and RAE content in inner and outer leaves of contrasting lettuce genotypes are shown in Table 1. The results are expressed in mg.100g^{-1} of initial concentration for each carotenoid and RAE in fresh leaves. The concentration of β-carotene was higher than lutein for all genotypes availed. In fresh raw lettuce leaves, there were no significant difference in lutein concentration between genotypes and age leaves. For β-carotene, the cultivar Dark Land had the highest concentration in inner leaves, 11.04 mg.100g^{-1} ($P < 0.001$) while the light green cultivars Grand Rapids and Verônica, along with internal leaves of 'Salinas 88' had the lowest concentrations, 7.18, 6.48 and 7.09 mg.100g^{-1}, respectively, suggesting a tendency towards smaller concentrations of this carotenoid in light green fresh leaves. This tendency is supported by the β-carotene concentration in outer leaves, that had 'Dark Land' and 'Salinas 88' with higher concentrations, 11.5 and 11.16 mg.100g^{-1}, respectively ($P < 0.05$). Also, the color difference between inner and outer leaves of 'Salinas 88' illustrates this trend. This cultivar was the only one with significant difference in β-carotene concentration between outer and inner leaves ($P < 0.05$), with outer leaves concentrating around 60% more β-carotene then inner ones. The RAE result followed the same trend of β-carotene and 'Dark Land' had the highest concentration in both inner and outer leaves, and 'Salinas 88' was the only cultivar with significant difference ($P < 0.05$) between different age leaves, with higher concentration in outer ones.

Table 1 Lutein, β-carotene and RAE concentrations in inner and outer leaves of contrasting lettuce genotypes

Genotype	Lutein mg/ 100g FW		β-carotene mg/ 100g FW		RAE mg/ 100g FW	
	Inner[1]	Outer	Inner	Outer	Inner	Outer
Dark Land	3.37 ± 0.16[a]	3.09 ± 0.3[a]	11.04 ± 2.6[a]	11.5 ± 0.81[a]	0.92 ± 0.22[a]	0.95 ± 0.06[a]
Dragoon	2.64 ± 0.28[a]	2.73 ± 0.11[a]	7.19 ± 1.04[b]	9.81 ± 0.58[ab]	0.59 ± 0.08[b]	0.81 ± 0.04[ab]
Grand Rapids	2.74 ± 0.44[a]	2.73 ± 0.06[a]	7.18 ± 1.13[b]	6.22 ± 0.12[ab]	0.59 ± 0.09[b]	0.65 ± 0.23[ab]
Parris Island	3.5 ± 0.28[a]	3.05 ± 0.16[a]	10.19 ± 0.62[ab]	8.93 ± 1.79[ab]	0.93 ± 0.14[ab]	0.7 ± 0.12[ab]
Salinas 88	2.96 ± 0.26[a]	3.01 ± 0.37[a]	7.09 ± 0.52[b]*	11.16 ± 1.41[a]	0.59 ± 0.04[b]*	0.93 ± 0.11[ab]
Verônica	2.48 ± 0.15[a]	2.38 ± 0.11[a]	6.48 ± 1.17[b]	5.49 ± 0.07[b]	0.54 ± 0.09[b]	0.45 ± 0[b]

1 Values are means ± SD of three biological replicates, each one being the pooled sample of at least three technical replicates, at P = 0.01 level. Similar letters for lutein, β-carotene and RAE in each leaf age are not significantly different (P = 0.01), using one-way ANOVA analysis followed by Tukey pos hoc test.

* Significant difference between inner and outer leaves for each cultivar.

In a previous lettuce study genotypes Dark Land, Parris Island and Salinas 88, were analysed for levels of β-carotene and lutein (Mou, 2005). Comparing with our results, in the firt study 'Dark Land' had a higher concentration of lutein and similar levels of β-carotene, 'Parris Island' had a similar concentration of lutein and a lower concentration of β-carotene and 'Salinas 88' presented the major difference between the two studies, displaing in the first study approximately ten fold lower levels of both carotenoids in the same leaf position. These differences between Mou (2005) and our study could be due the use of outer leaves, that had more intense green color than the internal leaves used by Mou (2005). Furthermore, in our study we used internal standard in the HPLC analyses to correct the carotenoid losses during extraction process, which may have contributed to highest concentration values estimated.

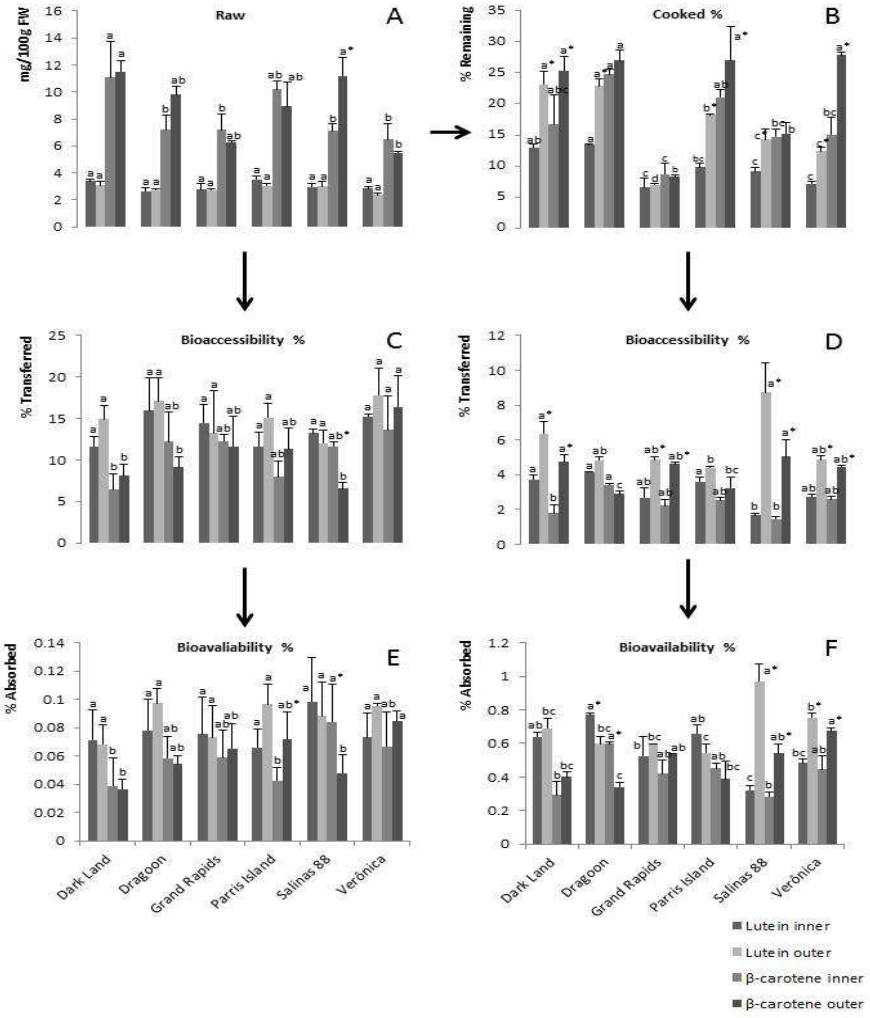
3.2 Effect of cooking in carotenoid concentration in inner and outer leaves in lettuce genotypes

The cooking effect over carotenoid concentration in lettuce genotypes is shown in Figure 2B. Generally, lutein was more sensitive to hydrothermal cooking than β-carotene. Lutein losses after cooking were more than 87 % considering the average of all genotypes, while β-carotene losses were 81 %. Using the same heat treatment of ours, a previous study with spinach showed losses after cooking of lutein and β-carotene of 58 % and 34 % respectively (Clotault et al., 2008). In another study that analyzed a mix of green leafy vegetables (without lettuce), the losses after cooking were more than 50 % for β-carotene (Mulokozi, Hedren, & Svanberg, 2004). Evidently, the losses in carotenoids during heat treatment depend on the kind of treatment applied. Moreover, the arrangement of carotenoids in the food matrix can results in sensibility differences between vegetables to hydrothermal cooking, which may affect the release of carotenoids from food matrix. In this sense, our results

suggest that lettuce can be more sensitive to heat treatment, resulting in lowers carotenoid concentrations after cooking.

In some lettuce genotypes analyzed the percentage of the initial amount of carotenoid remaining after cooking was higher than others. In raw lettuce, there was no significant difference for lutein content between genotypes (Table 1 and Fig. 2A). However, after cooking the percentage of remaining lutein varied among genotypes (Fig. 2B). The genotypes Dark Land and Dragoon had the highest percentage of lutein while 'Grand Rapids', 'Salinas 88' and 'Verônica' had the lowest concentration. Furthermore, excepted 'Grand Rapids' after cooking treatment all genotypes had significant difference in lutein concentration between inner and outer lettuce leaves, with higher lutein concentration in outer leaves ($P < 0.001$). For β-carotene, the differences in the pattern between genotypes also have changed from raw to cooked lettuce. 'Grand Rapids' had only 8 % of initial β-carotene remaining in both inner and outer leaves after cooking (the lowest concentration), followed by 'Salinas 88' with 14.9 %. Interestingly, in spite of the differences in β-carotene concentration observed between internal and external raw leaves of 'Salinas 88', this was not observed after cooking. On the other hand, 'Dark Land', 'Parris Island' and 'Verônica' that had no difference in initial β-carotene concentration between external and internal leaves, had significant difference between these leaves after cooking ($P < 0.01$, 0.05 and 0.001, respectively), with higher concentration in outer leaves. Moreover, 'Verônica' had the lowest concentration of β-carotene in raw leaves, but had the highest remaining percentage of this carotenoid in outer leaves after heat treatment. This percentage pattern between lettuce cultivars is the same of the results given in weight (data not shown). These results suggest a genotypic effect in lettuce food matrix stability, because the rank of lutein and β-carotene concentration in fresh leaves is not related to the rank of percentage remaining between lettuce genotypes after cooking.

Figure 2 Lutein and β-carotene profiles along cooking, digestion and absorption.



A: Lutein and β-carotene concentration in fresh raw inner and outer leaves of contrasting lettuce cultivars as measured by HPLC analysis; **B:** Lutein and β-carotene remaining in cooked lettuce leaves; **C:** Lutein and β-carotene transferred to micellar fraction after *in vitro* digestion of fresh raw lettuce leaves; **D:** Lutein and β-carotene transferred to micellar fraction after *in vitro* digestion of cooked lettuce leaves; **E:** Lutein and β-carotene absorbed by Caco-2 cells after *in vitro* digestion of fresh raw lettuce leaves; **F:** Lutein and β-carotene absorbed by Caco-2 cells after *in vitro* digestion of cooked lettuce leaves. Error bars are standard deviation of the mean from three biological replicates, each one being the pooled sample of at least three technical replicates, at $P = 0.01$ level. Bars with similar letters for lutein and β-carotene in each leafy age are not significantly different ($P = 0.01$), using one-way ANOVA analysis followed by Tukey pos hoc test. The * means significative difference between inner and outer leaves.

3.3 Carotenoid bioaccessibility from raw and cooked lettuce genotypes

The analysis of the carotenoids presented in the digested samples allows for an estimation of the effect of the *in vitro* digestion upon the release of carotenoids from the food matrix, while the analysis of the corresponding micellarized fraction gives information of which proportion of those released pigments are ready for the intestinal enterocyte absorption (Hornero-Méndez & Mínguez-Mosquera, 2007). Because of their hydrophobic nature, carotenoids in the mostly aqueous environment in plant foods must be transferred to bulk lipids or intestinal micelles in the digesta (Faulks & Southon, 2005). Previous studies indicated that the impact of digestion procedure on the recovery of carotenoids from the initial food was not damaging, implying that lutein and β-carotene are not destroyed during *in vitro* digestion (Clotault et al., 2008; Garrett et al., 2000; Rich et al., 2003).

After the *in vitro* digestion, micellar fraction was isolated from the digesta of raw and cooked lettuce leaves and the bioaccessibility, relative percentage of lutein and β-carotene transferred to micelar fraction was calculated for inner and outer leaves of each lettuce genotype (Figures 2C and 2D). The percentage transferred to aqueous fraction from raw lettuce was 14.36 % for lutein and 10.6 % for β-carotene, considering an average of all genotypes. For cooked lettuce, due to the decrease in concentration after cooking, only 4.38 % of initial lutein and 3.24 % of initial β-carotene presented in raw leaves were transferred to the micellar fraction. The lower bioaccessibility of carotenoids in cooked lettuce leaves is in agreement with a study conducted with savoy cabbage and broccoli between other green leafy vegetables (O'Sullivan et al., 2010), in which the cooking procedure reduced the micellarization of β-carotene. On the other hand in non-photosynthetic organs, cooking procedure increased the micellarization of carotenoids after *in vitro* digestion (Aherne et al., 2010; Hedren, Diaz, & Svanberg, 2002; Netzel et al., 2010; Veda, Platel, &

Srinivasan, 2008). This disparity between different kinds of vegetables organs in carotenoid release from food matrix after thermal processing, is dependent on the structural characteristics of the starting material (Lemmens, Van Buggenhout, Oey, Van Loey, & Hendrickx, 2009). In this sense, even though thermal treatment is related to disruption of food matrix, our results indicated a higher carotenoid transfer in digested raw samples than in cooked ones (Fig. 2C and 2D), which, according with literature, could be related to carotenoid degradation or isomerization after heat treatment (Aherne et al., 2010; Livny et al., 2003; Rodriguez-Amaya, 1999; Updike & Schwartz, 2003). However, if we consider the percentage transferred to micellar fraction based on the concentration remaining in cooked lettuce samples, there is an average of 35.81% for lutein and 19.59% for β-carotene (data not shown), which may suggests that the release in cooked samples was, in fact, more efficient that in raw ones. Interestingly, in spite of its lower initial concentration, 'Grand Rapids' had the highest release in this sense, reaching almost 70% for lutein and 55% for β-carotene in outer leaves (data not shown).

Considering the pattern regarding to carotenoid bioaccessibility between lettuce genotypes, there was no significant difference in the percent lutein transferred to micellar fraction in samples from raw lettuce and between different age leaves for each genotype. However, in cooked samples lutein percent transferred to micellar fraction was significant higher in outer leaves of 'Salinas 88' ($P < 0.001$) and in inner leaves 'Dragoon', 'Dark Land' and 'Parris Island' ($P < 0.01$) (Fig. 2D). Concerning the samples of different age leaves for each genotype, significant differences ($P < 0.001$) were detected for most of genotypes (excepting 'Dragoon' and 'Parris Island') with higher lutein transfer in outer leaves.

For β-carotene transfer, 'Verônica' was the cultivar with higher transfer from raw inner leaves after *in vitro* digestion and 'Dark Land' was the cultivar with lower transfer in both inner and outer leaves from raw

samples (Fig. 2C). Interestingly, ‘Dark Land’ had the highest β-carotene concentration in raw samples before *in vitro* digestion (Fig. 2A), while ‘Verônica’ had the higher transfer in both inner and outer leaves (Fig. 2C), despite the lowest initial concentration. Furthermore, the only significant difference ($P < 0.001$) between β-carotene transfer from raw inner and outer leaves was detected in ‘Salinas 88’, with higher transfer in inner leaves that had lowest initial β-carotene concentration in raw samples (Fig. 2A). These results suggest possible genotypic effects in the food matrix structure, because in raw leaves, higher initial β-carotene concentration seems to have a negative effect in β-carotene transfer to micellar fraction. In cooked samples, significant difference in β-carotene micellarization was also detected in both inner and outer leaves. In inner cooked leaves, the highest transfer was detected in ‘Dragoon’ ($P < 0.01$), while in outer leaves the highest transfer were in cultivars ‘Dark Land’ and ‘Salinas 88’ ($P < 0.001$). Curiously, the significant difference ($P < 0.001$) between β-carotene transfer in inner and outer leaves of ‘Salinas 88’ (Fig. 2D) has the same pattern of fresh raw leaves (Fig. 2A), which suggests a possible food matrix disruption in this cultivar after cooking, because this pattern was inverted in raw sample transfer (Fig. 2C).

3.4 Carotenoid bioavailability from raw and cooked lettuce genotypes

The release and absorption (bioavailability) of carotenoids by human body is a prerequisite for their nutritional impact. This can be strongly affected by the processing conditions used to prepare and disrupt the food matrix that contain them (Netzel et al., 2010). To determine the effect of processing on lutein and β-carotene bioavailability, the micellar fraction of the homogenized raw and cooked external and internal leaves of contrasting lettuce genotypes were placed onto a Caco-2 cell trans-well monolayer culture to mimic intestinal absorption.

The bioavailability results are disposed in the Table 2 and Figures 2E and 2F. The Caco-2 cells uptake of micelle samples from raw leaves was very low, with only 0.08 % (0.002 mg.100g^{-1}) of the initial lutein and 0.06 % (0.004 mg.100g^{-1}) of initial β-carotene absorbed in average of all genotypes (Table 2 and Figure 2E). However, these results are not surprising considering the complex arrangement and localization of carotenoids in the chloroplasts that form the food matrix on photosynthetic tissues. Moreover, despite the high concentration of carotenoids, the green leafy vegetables are essentially fat-free, which may be compromised the carotenoid absorption by intestinal epithelial. Absorption of carotenoids in humans occurs in a passive way and follows digestive pathways similar to those of lipids, which mean that protein or membrane bound carotenoids must be dissolved in a hydrophobic domain (oils, fats or bulk lipid emulsions) to increase the bioavailability (Faulks & Southon, 2005). In this sense, our results are in agreement with literature, because the meals prepared in the present study were fat-free and this could be prevented the carotenoids exposure to the putative bioactivity. Brown and colleagues also detected no absorption of carotenoids when salads with fat-free salad dressing were consumed and a substantially greater absorption of carotenoids was observed when salads were consumed with full-fat than with reduced-fat salad dressing (Brown et al., 2004).

Table 2 Lutein and β-carotene absorbed by Caco-2 cells after *in vitro* digestion of fresh and cooked inner and outer leaves of contrasting lettuce genotypes.

	Raw - mg.100g^{-1} FW			
	Lutein		**β-carotene**	
Genotype	**Inner[1]**	**Outer**	**Inner**	**Outer**
Dark Land	0.0023 ± 0.0006^{a}	0.002 ± 0.0002^{a}	0.0048 ± 0.001^{a}	0.0041 ± 0.0005^{a}
Dragoon	0.002 ± 0.0006^{a}	0.0026 ± 0.0003^{a}	0.0042 ± 0.001^{a}	0.0053 ± 0.0008^{a}
Grand Rapids	0.0019 ± 0.0002^{a}	0.0024 ± 0.0002^{a}	0.0039 ± 0.0005^{a}	0.0048 ± 0.0005^{a}
Parris Island	0.0022 ± 0.0003^{a}	0.0029 ± 0.0003^{a}	0.0046 ± 0.0006^{a}	0.0059 ± 0.0007^{a}
Salinas 88	0.0029 ± 0.001^{a}	0.0027 ± 0.0005^{a}	0.006 ± 0.002^{a}	0.0055 ± 0.001^{a}
Verônica	0.002 ± 0.0003^{a}	0.0022 ± 0.0001^{a}	0.0042 ± 0.0007^{a}	0.0046 ± 0.0003^{a}
	Cooked - mg.100g^{-1} FW			
	Lutein		**β-carotene**	
Genotype	**Inner**	**Outer**	**Inner**	**Outer**
Dark Land	0.022 ± 0.00009^{a}	0.021 ± 0.0004^{b}	0.045 ± 0.0001^{a}	0.045 ± 0.0007^{b}
Dragoon	0.019 ± 0.0001^{b}*	0.016 ± 0.0003^{d}	0.039 ± 0.0002^{b}*	0.034 ± 0.0007^{cd}
Grand Rapids	0.013 ± 0.00005^{c}*	0.016 ± 0.0004^{d}	0.028 ± 0.00003^{c}*	0.033 ± 0.0008^{d}
Parris Island	0.022 ± 0.00009^{a}*	0.016 ± 0.001^{d}	0.045 ± 0.0003^{a}*	0.033 ± 0.002^{d}
Salinas 88	0.0099 ± 0.00005^{d}*	0.029 ± 0.0005^{a}	0.02 ± 0.0001^{d}*	0.059 ± 0.001^{a}
Verônica	0.013 ± 0.00009^{c}*	0.017 ± 0.0001^{c}	0.028 ± 0.00008^{c}*	0.037 ± 0.0003^{c}

1 Values are absorption means ± SD of three biological replicates each one being the pooled sample of at least three technical replicates, at $P = 0.01$ level. Similar letters for lutein and β-carotene in each leafy age are not significantly different ($P = 0.01$), using one-way ANOVA analysis followed by Tukey pos hoc test.

* Significant difference between inner and outer leaves for each cultivar.

In cooked lettuce samples the percentage uptake by the cells was in average eight fold more efficient than in samples from raw lettuce, reaching 0.62 % (0.018 mg.100g^{-1}) for lutein and 0.44 % (0.037 mg.100g^{-1}) for β-carotene (Table 2 and Figure 2F). This rise in bioavailability from cooked lettuce samples is in agreement with the described in literature, once the disruption of food matrix by processing enhances the release of carotenoids and substantially improves their bioavailability in vegetables (Aherne et al., 2010; Nagao, 2014; Netzel et al., 2010). However, in our results the lutein and β-carotene transfer to micellar fraction from cooked samples was much lower than from raw samples, as described in the section 3.3 (Figures 2C and 2D). This compensation in absorption of carotenoids from cooked samples when comparing with raw samples is contrary with described in previous studies, in which an increase in carotenoid release from food matrix by cooking is directed related with higher absorption by the cells (Aherne et al., 2010; Nagao, 2014; Netzel et al., 2010).

Our results indicate some barrier acting in micelles from fresh leaves that, despite the higher transfer, rate were not absorbed by cells. Heat treatment by cooking procedure resulted in a lower carotenoid transfer from food matrix, but an increase in absorption was detected, which suggests a better specificity between carotenoids micellarizated from cooked lettuce leaves and cells monolayer. These results can be explained by some factor that could be related to bioavailability efficiency. According with previous studies, the food processing activities, such as thermal processing, mincing or liquefying can result in changes to carotenoid chemistry, probably through isomerization or oxidation reactions (Livny et al., 2003; Rodriguez-Amaya, 1999; Updike & Schwartz, 2003). Processing activities usually increase bioavailability through increased release of bound carotenoids from the food matrix, but when this processing is by thermal treatment, degradations in carotenoid chemistry can adversely affect bioavailability in some food crops. Also, the low carotenoid

solubility in digestive fluid can be related to the low bioavailability (Lima et al., 2012). The solubility and location of carotenoids in the liquid digestion phase can vary in function of their polarity. For instance, the xanthophylls (lutein group) are more polar than carotenes (β-carotene). In this sense, the β-carotene location in the liquid digestion phase is in the triglyceride nucleus (hydrophobic region) while lutein is in the liquid face (hydrophilic). This carotenoid location can explain why lutein is more absorbed than β-carotene (Figures 1E and 1F), once outer liquid components spontaneously give off and migrate to micelle mix of bile salts on duodenum, while nucleus components need triglyceride digestion before absorption. Consequently, xanthophyll can be more readily absorbed than carotenes by enterocyte membrane and, subsequently by chylomicrons, what increase their bioavailability (Lima et al., 2012).

The results regarding to bioavailability between lettuce genotypes presented no significant difference for lutein absorption in samples from raw leaves, following the same pattern of fresh leaves (Table 1, Table 2 and Figures 2A and 2E), which probably is related to the absorption inhibition that resulted in the same absorption raters for all genotypes, no matter how much was the initial lutein concentration. Also for β-carotene, despite the significant difference between genotypes observed in percentage from fresh leaves ($P < 0.001$), no difference in concentration was detected between them (Table 2) even though some genotypes presented differences in percentage absorbed by cells (Figure 2E). On the other hand, in cooked samples significant differences ($P < 0.001$) were detected between genotypes in lutein uptake by cells (Table 2 and Figure 2F). Genotypes Dark Land, Parris Island and Dragoon had the higher lutein absorption rates in inner leaves while ‘Grand Rapids’, ‘Salinas 88’ and ‘Verônica’ had the lower absorption in same leaf position. In outer leaf samples, ‘Salinas 88’ had the highest lutein absorption, while ‘Dragoon’, ‘Grand Rapids’ and ‘Parris Island’ had the lowest absorption. Considering β-carotene uptake, also a significant difference

between lettuce genotypes was detected ($P < 0.001$). ‘Dark Land’ and ‘Parris Island’ were the cultivars that had more β-carotene from inner leaves absorbed by the cells and ‘Dark Land’ along with ‘Salinas 88’ had the higher values for outer leaves (Table 2). The pattern of lutein and β-carotene absorption by Caco-2 cells had the same ranking of genotypes observed in micellar fraction of cooked samples for lutein (Fig. 2D and 2F) and the same pattern of fresh leaves and cooked samples micellar for β-carotene (Fig. 2A, 2D and 2F).

The genotypic effect observed in lutein and β-carotene concentration in fresh lettuce leaves, seems to be not related to the real absorption by the intestinal epithelial, resulting in the same level of absorption of these two carotenoids when lettuce is consumed raw. If the food matrix is not disrupted, a higher initial carotenoid concentration does not result in an increase in carotenoid absorption by intestinal cells. However, when a cooking procedure is applied, the lutein and β-carotene genotypic effect can be observed and a relation between initial carotenoid concentrations seems be related to final intestinal absorption. In this sense, a higher initial carotenoid concentration in lettuce leaves does not result in a higher functional effect in human body, unless the matrix food is disrupted.

4 CONCLUSIONS

The results of the present study suggest that the differences between lettuce cultivars in lutein and β-carotene concentrations in inner and outer fresh leaves are not directed related to the absorption and transport of these carotenoids by human intestine. Disruption of the food matrix by cooking lettuce leaves resulted in losses of carotenoid concentration and in reduction of carotenoid transfer to micellar fraction, but it was compensated by an eight fold raise in bioavailability of lutein and β-carotene comparing with the absorption of

digestion of fresh leaves, which may increase the antioxidant activity. Unless the matrix food is disrupted by heat treatment, the absorption of carotenoids present in biofortified lettuce occurs in a very low rate, similar to that of low carotenoid cultivars. Considering the lutein and β-carotene absorbed by Caco-2 cells, when the food matrix was broken, 'Dark Land' was the more efficient cultivar, along with external leaves of 'Salinas 88'. In this sense, our results propose that the development and breeding programs of carotenoid biofortified leaf crops must be related to efforts to increase the bioavailability of carotenoids present in food matrix.

ACKNOWLEDGEMENTS

The authors wish to thank the Coordenação de Pessoal de Nível Superior of Brazil Ministry of Education (CAPES – BRAZIL) and Science Fundation Ireland (SFI) that provided the support necessary for this study. We are grateful to Dr. Aoife Boyd from the Microbiology Deparment at the National University of Ireland Galway for provide the cells , to Gerard Fahy from the Chemistry Department for technical support and to lettuce breeding program from the Agricultural Department at Universidade Federal de Lavras for providing seeds.

REFERENCES

Águila Ruiz-Solaa, M., & Rodríguez-Concepcióna, M. (2012). Carotenoid Biosynthesis in Arabidopsis: A Colorful Pathway. *The Arabdopsis Book, 10*(e0158), 28. doi: 10.1199/tab.0158

Aherne , S. A., Daly, T., Jiwan, M. A., O'Sullivan, L., & O'Brien, N. M. (2010). Bioavailability of b-carotene isomers from raw and cooked carrots using an in vitro digestion model coupled with a human intestinal Caco-2 cell model. *Food Research International, 43*, 1449-1454. doi: 10.1016/j.foodres.2010.04.026

Ampomah-Dwamena, C., Dejnoprat, S., Lewis, D., Sutherland, P., Volz, R. K., & Allan, A. C. (2012). Metabolic and gene expression analysis of apple (Malus x domestica) carotenogenesis. *J Exp Bot, 63*(12), 4497-4511. doi: 10.1093/jxb/ers134

Ampomah-Dwamena, C., McGhie, T., Wibisono, R., Montefiori, M., Hellens, R. P., & Allan, A. C. (2009). The kiwifruit lycopene beta-cyclase plays a significant role in carotenoid accumulation in fruit. *J Exp Bot, 60*(13), 3765-3779. doi: 10.1093/jxb/erp218

Brown, M. J., Ferruzzi, M. G., Nguyen, M. L., Cooper, D. A., Eldridge, A. L., Schwartz, S. J., & White, W. S. (2004). Carotenoid bioavailability is higher from salads ingested with full-fat than with fat-reduced salad dressings as measured with electrochemical detection. *Am J Clin Nutr, 80*(2), 396-403.

Campos, F. M., & Rosado, G. P. (2005). Novos Fatores de Conversão de Carotenóides Provitamínicos A. *Ciência e Tecnologia de Alimentos*, 571-578.

Cazzonelli, C. I., Yin, K., & Pogson, B. J. (2009). Potential implications for epigenetic regulation of carotenoid biosynthesis during root and shoot development. *Plant Signal Behav, 4*(4), 339-341.

Clotault, J., Peltier, D., Berruyer, R., Thomas, M., Briard, M., & Geoffriau, E. (2008). Expression of carotenoid biosynthesis genes during carrot root development. *J Exp Bot, 59*, 3563-3573. doi: 10.1093/jxb/ern210

Courraud, J., Berger, J., Cristol, J. P., & Avallone, S. (2013). Stability and bioaccessibility of different forms of carotenoids and vitamin A during in vitro digestion. *Food Chem, 136*(2), 871-877. doi: 10.1016/j.foodchem.2012.08.076

Eastwood, M., & Kritchevsky, D. (2005). Dietary fiber: how did we get where we are? *Annu Rev Nutr, 25*, 1-8. doi: 10.1146/annurev.nutr.25.121304.131658

Ellwood, K. C., Chatzidakis, C., & Failla, M. L. (1993). Fructose utilization by the human intestinal epithelial cell line, Caco-2. *Proc Soc Exp Biol Med, 202*(4), 440-446.

Faulks, R. M., & Southon, S. (2005). Challenges to understanding and measuring carotenoid bioavailability. *Biochim Biophys Acta, 1740*(2), 95-100. doi: 10.1016/j.bbadis.2004.11.012

Finley, J. W. (2005). Proposed criteria for assessing the efficacy of cancer reduction by plant foods enriched in carotenoids, glucosinolates, polyphenols and selenocompounds. *Ann Bot, 95*(7), 1075-1096. doi: 10.1093/aob/mci123

Garcia, A. L., Ruhl, R., Herz, U., Koebnick, C., Schweigert, F. J., & Worm, M. (2003). Retinoid- and carotenoid-enriched diets influence the ontogenesis of the immune system in mice. *Immunology, 110*(2), 180-187.

Garrett, D. A., Failla, M. L., & Sarama, R. J. (2000). Estimation of carotenoid bioavailability from fresh stir-fried vegetables using an in vitro digestion/Caco-2 cell culture model. *J Nutr Biochem, 11*(11-12), 574-580.

Ghavami, A., Coward, W. A., & Bluck, L. J. (2012). The effect of food preparation on the bioavailability of carotenoids from carrots using intrinsic labelling. *Br J Nutr, 107*(9), 1350-1366. doi: 10.1017/S000711451100451X

Granado, F., Olmedilla, B., & Blanco, I. (2003). Nutritional and clinical relevance of lutein in human health. *Br J Nutr, 90*(3), 487-502.

Grusak, M. A., & DellaPenna, D. (1999). Improving the Nutrient Composition of Plants to Enhance Human Nutrition and Health1. *Annu Rev Plant Physiol Plant Mol Biol, 50*, 133-161. doi: 10.1146/annurev.arplant.50.1.133

Hedren, E., Diaz, V., & Svanberg, U. (2002). Estimation of carotenoid accessibility from carrots determined by an in vitro digestion method. *Eur J Clin Nutr, 56*(5), 425-430. doi: 10.1038/sj.ejcn.1601329

Hornero-Méndez, D., & Mínguez-Mosquera, M. I. M. (2007). Bioaccessibility of carotenes from carrots: Effect of cooking and addition of oil. *Sciene Direct, 8*, 407-412. doi: 10.1016/j.ifset.2007.03.014

Johnson, E. J., Hammond, B. R., Yeum, K. J., Qin, J., Wang, X. D., Castaneda, C., . . . Russell, R. M. (2000). Relation among serum and tissue concentrations of lutein and zeaxanthin and macular pigment density. *Am J Clin Nutr, 71*(6), 1555-1562.

Kopsell, D. A., Kopsell, D. A., & Curran-Celentano, J. (2005). Carotenoids and Chlorophyll Pigments in Sweet Basil Grown in the Field and Greenhouse. *HortScience, 40*, 1230-1233.

Kopsell, D. A., Kopsell, D. E., Lefsrud, M. G., Curran-Celentano, J., & Dukach, E. (2004). Variation of Lutein, β-Carotene, and Chlorophyll concentrations among Brassica oleraceae cultigens and seasons. *HortScience, 39*, 361-364.

Lemmens, L., Van Buggenhout, S., Oey, I., Van Loey, A., & Hendrickx, M. (2009). Towards a better understanding of the relationship between the beta-carotene in vitro bioaccessibility and pectin structural changes: A case study on carrots. *Food Research International, 42*, 1323-1330.

Lima, J. P., Lopes, C. O., Dias, N. A. A. & Angelis, M. C. (2012). Atividade e biodisponibilidade dos carotenóides no organismo. *Revista Ciências em Saúde, 2*, 65-73.

Livny, O., Reifen, R., Levy, I., Madar, Z., Faulks, R., Southon, S., & Schwartz, B. (2003). Beta-carotene bioavailability from differently processed carrot meals in human ileostomy volunteers. *Eur J Nutr, 42*(6), 338-345. doi: 10.1007/s00394-003-0430-6

Mortensen, A., Skibsted, L. H., & Truscott, T. G. (2001). The interaction of dietary carotenoids with radical species. *Arch Biochem Biophys, 385*(1), 13-19. doi: 10.1006/abbi.2000.2172

Mou, B. (2005). Genetic Variation of Beta-carotene and Lutein Contents in Lettuce. *Journal of the American Society for Horticultural Science, 130*, 7.

Mulokozi, G., Hedren, E., & Svanberg, U. (2004). In vitro accessibility and intake of beta-carotene from cooked green leafy vegetables and their estimated contribution to vitamin A requirements. *Plant Foods Hum Nutr, 59*(1), 1-9.

Nagao, A. (2014). Bioavailability of Dietary Carotenoids: Intestinal Absorption and Metabolism. *JARQ - Japan Agricultural Research Quarterly, 48*, 385-391.

Netzel, M., Netzel, G., Zabaras, D., Lundin, L., Day, L., Addepalli, R., . . . Seymour, R. (2010). Release and absorption of carotenes from processed carrots (Daucus carota) using in vitro digestion coupled with a Caco-2 cell trans-well culture model. *Food Research International, 44*, 868-874. doi: 10.1016/j.foodres.2010.10.058

O'Connell, O. F., Ryan, L., & O'Brien, N. M. (2007). Xanthophyll carotenoids are more bioaccessible from fruits than dark green vegetables. *Sciene Direct, 27*, 258-264. doi: 10.1016/j.nutres.2007.04.002

O'Sullivan, L., Galvin, K., Aherne, S. A., & O'Brien, N. M. (2010). Effects of cooking on the profile and micellarization of 9-cis-, 13-cisand all-trans-b-carotene in green vegetables. *Food Research International, 43*, 1130-1135. doi: 10.1016/j.foodres.2010.02.012

Rich, G. T., Bailey, A. L., Faulks, R. M., Parker, M. L., Wickham, M. S., & Fillery-Travis, A. (2003). Solubilization of carotenoids from carrot juice and spinach in lipid phases: I. Modeling the gastric lumen. *Lipids, 38*(9), 933-945.

Rodriguez-Amaya, D. B. (1999). Changes in carotenoids during processing and storage of foods. *Arch Latinoam Nutr, 49*(3 Suppl 1), 38S-47S.

Seifried, H. E., McDonald, S. S., Anderson, D. E., Greenwald, P., & Milner, J. A. (2003). The antioxidant conundrum in cancer. *Cancer Res, 63*(15), 4295-4298.

Sommerburg, O., Keunen, J. E., Bird, A. C., & van Kuijk, F. J. (1998). Fruits and vegetables that are sources for lutein and zeaxanthin: the macular pigment in human eyes. *Br J Ophthalmol, 82*(8), 907-910.

Stahl, W., van den Berg, H., Arthur, J., Bast, A., Dainty, J., Faulks, R. M., . . . Astley, S. B. (2002). Bioavailability and metabolism. *Mol Aspects Med, 23*(1-3), 39-100.

Tang, L., Jin, T., Zeng, X., & Wang, J. S. (2005). Lycopene inhibits the growth of human androgen-independent prostate cancer cells in vitro and in BALB/c nude mice. *J Nutr, 135*(2), 287-290.

Tanumihardjo, S. A., Palacios, N., & Pixley, K. V. (2010). Provitamin a carotenoid bioavailability:what really matters? *Int J Vitam Nutr Res, 80*(4-5), 336-350. doi: 10.1024/0300-9831/a000042

Thakkar, S. K., Maziya-Dixon, B., Dixon, A. G., & Failla, M. L. (2007). Beta-carotene micellarization during in vitro digestion and uptake by Caco-2 cells is directly proportional to beta-carotene content in different genotypes of cassava. *J Nutr, 137*(10), 2229-2233.

Tyssandier, V., Reboul, E., Dumas, J. F., Bouteloup-Demange, C., Armand, M., Marcand, J., . . . Borel, P. (2003). Processing of vegetable-borne carotenoids in the human stomach and duodenum. *Am J Physiol Gastrointest Liver Physiol, 284*(6), G913-923. doi: 10.1152/ajpgi.00410.2002

Updike, A. A., & Schwartz, S. J. (2003). Thermal processing of vegetables increases cis isomers of lutein and zeaxanthin. *J Agric Food Chem, 51*(21), 6184-6190. doi: 10.1021/jf030350f

van Lieshout, M., West, C. E., Muhilal, Permaesih, D., Wang, Y., Xu, X., . . . Lugtenburg, J. (2001). Bioefficacy of beta-carotene dissolved in oil studied in children in Indonesia. *Am J Clin Nutr, 73*(5), 949-958.

Veda, S., Platel, K., & Srinivasan, K. (2008). Influence of food acidulants and antioxidant spices on the bioaccessibility of beta-carotene from selected vegetables. *J Agric Food Chem, 56*(18), 8714-8719. doi: 10.1021/jf801374d

Yonekura, L., & Nagao, A. (2009). Soluble fibers inhibit carotenoid micellization in vitro and uptake by Caco-2 cells. *Biosci Biotechnol Biochem, 73*(1), 196-199. doi: 10.1271/bbb.80510