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Research article

Carotenoid profiling and the expression of carotenoid biosynthetic genes in developing coffee grain

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ABSTRACT

Roasted coffee contains a complex array of volatile organic compounds (VOCs) which make an important contribution to the characteristic flavour and aroma of the final beverage. It is thought that a few of the potent coffee aroma components, such as "β-damascenone", could be derived from carotenoid precursors. In order to further investigate the potential link between carotenoids and coffee aroma profiles, we have measured the carotenoid content in developing coffee grain. The data obtained confirms the presence of lutein in the grain, and additionally shows that the immature coffee grain also contains significant amounts of β-carotene, α-carotene, violaxanthin, and neoxanthin. Complimentary quantitative gene expression analysis revealed that all the carotenoid biosynthetic genes examined are expressed in the grain, and that the transcript levels are gene and stage dependent. Furthermore, consistent with the reduction of the carotenoid levels at the last stage of grain development (mature-red), most of the transcript levels were also found to be lower at the final developmental stage. Quantitative expression analysis of the carotenoid genes was also carried out for the developing pericarp tissue of the coffee cherries. Again, all the genes examined were expressed, and in most cases, the highest transcript levels were detected around the large green–yellow stages, a period when carotenoid synthesis is probably greatest.

1. Introduction

Carotenoids have been found in all plants, as well as in a wide range of algae, certain fungi and bacteria [1], and represent the largest group of pigments in nature, with some 600 different carotenoids identified to date (for review see Cunningham and Gantt [2]). Added to this diversity of compounds, are carotenoid derived volatile and non-volatile organic compounds called apocarotenoids. These apocarotenoids, which can be produced from carotenoids by either enzymatic [3] or non-enzymatic [4] mechanisms, constitute another important set of molecules in nature. In green tissues of plants, the main function of the carotenoids is to protect the photosynthetic apparatus from high light [5]. Carotenoids also accumulate in other plant tissues, where they can help to attract pollinators and aid in seed dispersion via their strong red, orange and yellow colours. Functionally, different apocarotenoids have been found to influence plant development, to possess antifungal activities, and to have animal attracting scents [6]. Many of the functionalities of carotenoids and apocarotenoids, such as antioxidant properties, colour, and aromatic quality also make these compounds essential and valuable constituents plant derived foods [7].

Many of the details of the carotenoid biosynthesis pathway in key model plants such as tomato are known (for review of carotenoid biosynthesis see Cunningham and Gantt [2]). Carotenoids synthesized by this pathway can be cleaved by a number of specific enzymes in the plant, generating physiologically important molecules such as the phytohormone ABA [8]. Carotenoids are also cleaved by the enzymes such as the carotenoid cleavage dioxygenase 1 (*CCD1*) [3,9] and CCD4 [10], resulting in the formation of a number of apocarotenoids, including some that have significant aroma characteristics and have very low odor thresholds (for example, odor thresholds of β-ionone and geranylacetone are 0.007

Abbreviations: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; PTOX, plastid terminal oxidase; LCY-B, lycopene β-cyclase; LYC-E, lycopene ε-cyclase; CRTR-B, β-carotene hydroxylase; CRTR-E, ε-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violanthin de-epoxidase; NYS, neoxanthin synthase; CCD1, carotenoid cleavage dioxygenase 1; NCED3, 9-cis-epoxycarotenoid cleavage dioxygenase 3.

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and 60 nl l^{-1}, respectively [11]). To explore carotenoid metabolism in coffee, we have recently characterized several cDNA sequences representing genes involved in carotenoid metabolism, and studied the expression of these genes in robusta (*C. canephora*) leaves [12] Table 1 indicates the frequency with which these different sequences appear within our coffee EST libraries.

The flavour and aroma of coffee results from a complex array of volatile organic compounds (VOCs) and non-volatile organic compounds, and to date, over 800 of such compounds have been detected in the final beverage [13]. It is known that many of the aroma and flavour molecules formed during roasting are products of Maillard type reactions between molecules containing amino groups and carbonyl groups [14]. However, other chemical reactions induced by roasting have recently begun to attract attention. For example, the flavour active compound 1-methylpyridine, found in relatively high quantities in roasted coffee, is proposed to be a roasting induced break-down product of the alkaloid trigonelline in green coffee [15,16]. Similarly, β-damascenone, a very potent and volatile aroma compound found in both low levels in green coffee [1718] and at higher levels in roasted coffee [18] is thought to be formed primarily by heat/chemical induced degradation of one or more carotenoids [18,19].

Although plant carotenoids are widely studied, little detailed attention has been given to the functions of the carotenoids found in plant seeds. It is currently believed that seed carotenoids assist in protecting the membranes and the photosynthetic apparatus of chlorophyll containing seeds [20], as well as being precursors for plant growth and development regulators such as ABA and strigolactones. In coffee, the carotenoids and apocarotenoids found in the grain may have a further utility, i.e. acting as a pool of coffee VOCs precursors. Given that the coffee grain carotenoids have both clear physiological roles, and a proposed quality role, we decided to study the accumulation of these metabolites in the developing grain. Here, we show that several carotenoids can be clearly detected in the later stages of the developing grain, but that they undergo significant breakdown towards the final stage of maturation. In addition, we found that coffee grain accumulates both α- and β-carotene, confirming our earlier analysis which showed coffee leaves accumulated both carotenoids. In order to begin to understand how the synthesis and degradation of carotenoids and apocarotenoids are controlled during grain development, we quantified the expression levels of a set of recently characterized coffee carotenoid genes [12] during several grain development stages, as well as during pericarp development. Finally, we discuss the data obtained regarding the importance of the coffee carotenoids in grain development and the proposal that coffee carotenoids represent part of the pool of precursors for roasted coffee VOCs.

Table 1
Number and distribution of ESTs in the *C. canephora* unigene.

	Number of ESTs						
	Unigene	Grain 30w	46w	Pericarp	Leaf	Total	NCBI[a]
PSY	123321	**2**	0	**1**	0	**3**	DQ157164
PDS	ND[b]	0	0	0	0	0	DQ357179
ZDS	ND	0	0	0	0	0	DQ357180
PTOX	121182	0	0	0	**3**	**3**	DQ233245
LYC-E	131043	0	0	**1**	0	**1**	DQ357178
CRTR-B	123117	**1**	**1**	**3**	**3**	**8**	DQ157165
ZEP	112969	0	0	**2**	**1**	**3**	DQ357177
VDE	130454	0	0	**1**	0	**1**	DQ233246
NCED3	130641		0	**1**	0	**1**	DQ157167
CCD1	121850	**3**	0	0	0	**3**	DQ157166
FIB1	119688	0	**1**	**1**	0	**2**	DQ157168

PSY, phytoene synthase; *PTOX*, plastid terminal oxidase; *CcLYC-E*, lycopene ε-cyclase; *CRTR-B*, β-carotene hydroxylase; *ZEP*, zeaxanthin epoxidase; *VDE*, violaxanthin de-epoxidase; *NCED3*, 9-cis-epoxycarotenoid dioxygenase; *CCD1*, carotenoid cleavage dioxygenase 1; *FIB*, fibrillin.

[a] NCBI accession numbers of the Coffea sequences are shown.

[b] ND – not detected.

2. Materials and methods

2.1. Plant material and treatment

Freshly harvested fruit at different stages of development [small green fruit (SG), large green fruit (LG), yellow fruit (YG) and red fruit (RG)] were harvested from *Coffea arabica* L. cv. Caturra T2308 grown under greenhouse conditions (25 °C, 70% RH) in Tours, France, and from *Coffea canephora* (robusta) cv. BP409 grown in the field at the Indonesian Coffee and Cacao Research Center (ICCRI), Indonesia. *Coffea canephora* cv. FRT05 and cv. FRT64 cherries were harvested from field grown trees in Equator. Fresh tissues were frozen immediately in liquid nitrogen and then stored at −80 °C until used for RNA extraction. The preparation and analysis of the EST libraries generated from leaves, grain and pericarp tissues of *C. canephora* coffee have been described previously [21].

For the germination studies, cherries were collected from *Coffea arabica* L. cv. Caturra T2308 grown under greenhouse conditions and processed as described by Simkin et al. (2006). Germinating samples for analysis were collected at T0 and at, 3, 5, 30 and 60 days after imbibition (DAI). The emerging radicle and any cotyledonous material present were removed and only the grain was used for RNA extraction.

2.2. Carotenoid extraction and analysis

The method used to analyse and quantify carotenoids from *C. arabica* (T2308) and *C. canephora* (FRT05) grain is detailed in ref. [22]. Briefly, eight to nine grain were ground in liquid nitrogen and freeze dried. Carotenoids were extracted from 100 mg of freeze dried material using methanol:chloroform (1:3 by vol) and partitioned against 50 mM Tris–HCl pH 7.0 (2 vols). Astaxanthin was added to the coffee samples prior to extraction to normalise the results obtained. HPLC separations were performed on a C18 reverse-phase column (Macherey–Nagel). The solvent consisted initially of 85% acetonitrile/methanol (75:25) and 15% water, followed by a gradient decreasing water content to 8% in 12 min, to 5% over the next 10 min and then to 0% over the next 3 min. 100% acetonitrile/methanol (75:25) was then kept until the end of the run. Replications were carried out on two to three sets of eight to nine grain from the same sample batch.

These same samples were also separated on a C30 reverse-phase column (250 × 4.6 mm) manufactured by YMC Co. Ltd and purchased from Interchim (France) using the method detailed in Fraser et al. [23]. The mobile phases used were: methanol (A), water/methanol (20/80 by vol) containing 0.2% ammonium acetate (B) and tert-methyl butyl ether (C). The gradient used was 95% A/5% B isocratically for 12 min, a step to 80% A/5% B and 15% C at 12 min, followed by a linear gradient to 30% A/5% B/65% C by 30 min. Quantification was achieved from dose-response curves and identification of carotenoids was achieved by co-chromatography and by comparing the spectral properties acquired on-line with standards.

2.3. Extraction of total RNA and RT-PCR

Samples were ground into a powder and total RNA was extracted from this powder using the method described previously [24]. Using oligo (dT_{20}) as a primer, cDNA was prepared from approximately 4 μg total RNA according to the protocol in the