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An investigation of carotenoid biosynthesis in *Coffea canephora* and *Coffea arabica*

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Summary

Carotenoids are essential components of the photosynthetic apparatus in a wide range of organisms. They participate in the adaptation of plastids to changing environmental light conditions and prevent photo-oxidative damage of the photosynthetic apparatus by detoxifying reactive oxygen species. We identified eight cDNAs from the carotenoid biosynthetic pathway (*PSY*, *PDS*, *ZDS*, *PTOX*, *LCY-E*, *CRTR-B*, *ZEP* and *VDE*) and two cDNA encoding carotenoid cleavage dioxygenase family members (*NCED3* and *CCD1*) in *Coffea canephora*. We also obtained cDNA encoding several different fibrillin proteins involved in carotenoid sequestration (FIB). Expression of the coffee carotenoid genes was determined in leaf, branch and flower tissues using quantitative RT-PCR. Expression analysis of these genes in leaf tissue from osmotically stressed plants was also carried out. These experiments showed that the transcript levels of *PTOX*, *CRTR-B*, *NCED3*, *CCD1* and *FIB1* increased under these stress conditions, while *LCY-E* decreased, indicating that the metabolic flux towards the xanthophyll cycle branch of the carotenoid biosynthetic pathway may be favoured in leaves under drought conditions. Functional analysis of CcCRTR-B using an *in vivo* method employing *Escherichia coli* strains engineered to make carotenoids confirmed that the β-carotene hydroxylase activity of CcCRTR-B generates β-cryptoxanthin and zeaxanthin from β-carotene. A similar approach was also used to show that *CcCCD1* encoded a functional 9,10(9′10′) carotenoid cleavage dioxygenase, and thus that this enzyme is capable of forming one or more

Abbreviations: Ca, *Coffea arabica*; Cc, *Coffea canephora*; CRTISO, carotenoid isomerase; CRTR-B, β-carotene hydroxylase; CRTR-E, ε-carotene hydroxylase; LCY-B, lycopene β-cyclase; LCY-E, lycopene ε-cyclase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; NXS, neoxanthin synthase; PDS, phytoene desaturase; PSY, phytoene synthase; PTOX, plastid terminal oxidase; VDE, Violaxanthin De-Epoxidase; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase

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apocarotenoids *in vivo*. Finally, high-performance liquid chromatography analysis of coffee leaves revealed the presence of α-carotene and suggests that *Coffea arabica* may have higher levels of α-carotene than *C. canephora*.

Introduction

Carotenoids have been identified in all plants examined, a wide range of algae and in certain fungi and bacteria (Goodwin, 1980; Fraser et al., 1999). Carotenoids carry out several important functions, such as the stabilization of lipid membranes (Havaux, 1998), and light collection for photosynthesis, as well as protecting the photosystem from photo-oxidation by reactive oxygen species produced by the excited triplet state of chlorophyll during photosynthesis (Frank and Cogdell, 1996; Ledford and Niyogi, 2005). The protective function of carotenoids within the photosystem is so critical that a block in carotenoid biosynthesis induced by a mutation such as the tomato Ghost mutation (Josse et al., 2000), or by herbicides such as norflurazon (Simkin et al., 2000), results in severe photobleaching (Sandmann and Böger, 1989). The carotenoid biosynthetic pathway has been intensely studied since the early 1960s (for review see Sandmann et al., 2006). While carotenoids are synthesized within the plastid compartment of the cell, the corresponding genes are located in the nucleus and their protein products are imported into the chloroplast. Chloroplasts accumulate β-carotene and xanthophylls, which are primarily located in the photosynthetic membranes where they have been found to be associated with the light-harvesting complexes and reaction centres (Thayer and Björkman, 1992; Ruban et al., 1994; Ruban et al., 1999; Telfer, 2002).

The carotenoid biosynthetic pathway is presented in Figure 1. The first true carotenoid is formed by the condensation of two molecules of geranylgeranyl diphosphate into phytoene catalysed by phytoene synthase (PSY; EC 2.5.1.32). Phytoene then undergoes four desaturation steps, which require the plastid terminal oxidase (PTOX) as a co-factor, resulting in the formation of lycopene (Cunningham and Gantt, 1998; Kuntz, 2004). Lycopene then serves as a substrate for the formation of both α- and β-carotene via two cyclization reactions (Cunningham et al., 1996; Ronen et al., 1999). β-carotene can then be converted to zeaxanthin by two successive hydroxylation steps, while α-carotene is converted in two steps to lutein (Sandmann, 1994; Tian et al., 2004). Zeaxanthin is epoxidized by the enzyme zeaxanthin epoxidase (ZEP) in two steps to give antheraxanthin and violaxanthin (Bouvier et al., 1996; Marin et al., 1996). Finally, the last step in carotenoid synthesis in higher plants is the formation of neoxanthin from violaxanthin by a reaction catalysed by neoxanthin synthase (NXS; Al-Babili et al., 2000; Bouvier et al., 2000). In high light, violaxanthin (V) can be converted back into antheraxanthin (A) and zeaxanthin (Z) by the activity of violaxanthin de-epoxidase (VDE).

The inter-conversion of V and Z is known as the xanthophyll cycle, and this cycle is implicated in the adaptation of plastids to changing environmental light conditions (for review see Hieber et al., 2000). In low light or darkness, the thylakoid proton gradient is small and epoxidation of Z to V is favoured, resulting in accumulation of V and a net loss of Z. In contrast, high light conditions promote the conversion of V to Z, resulting in an increase in zeaxanthin (Ruban et al., 1994; Färber et al., 1997). The ratio of Z to V, which reflects environmental conditions, is referred to as the de-epoxidation state (DEPS). A number of stress conditions that can increase the level of reactive oxygen species, such as wounding, cold and salt stress, have been shown to cause an increase in DEPS. For example, with the onset of drought, an increase in zeaxanthin was observed in the Rice cv. Araure 4, resulting in a 40% increase in DEPS (Pieters and El Souki, 2005). The DEPS state was also shown to increase under drought conditions in *Talinum triangulare* (Pieters et al., 2003). In recent years, a gene family representing 10–13 independent amino acid sequences belonging to the fibrillin family has been identified in *Arabidopsis*, tomato and rice (Laizet et al., 2004). One of these, AtFIB1 and its orthologues in pepper (FIB1; Chen et al., 1998; Simkin et al., 2000), and potato (CDSP34; Gillet et al., 1998), have been shown to be strongly induced under photo-oxidative stress conditions. Langenkämper et al. (2001) also demonstrated that *FIB1* transcripts accumulate in the leaves of plants such as tobacco, tomato, *Arabidopsis* and barley under conditions of water deficit.

Coffee is one of the three most important commodities in the international agriculture trade