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The carotenoid dioxygenase gene family in maize, sorghum, and rice

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ABSTRACT

Carotenoids and their apocarotenoid derivatives play essential physiological and developmental roles and provide plants tolerance to a variety of stresses. Carotenoid cleavage dioxygenases mediate the degradation of carotenoids to apocarotenoids. A better understanding of biosynthesis vs. degradation could be useful for controlling carotenoid levels leading to improved plant fitness and/or enhanced content of nutritionally valuable carotenoids. The Poaceae (grass) plant family contains many crops of agronomic value. Therefore this study focused on characterizing the carotenoid dioxygenase gene family in the grass species maize, rice, and sorghum with comparison made to newly identified gene families in two non-seed plants as well as an alga and previously identified eudicot genes. Genome analysis was used to map grass genes encoding the carotenoid dioxygenases to chromosome locations. Sequences of encoded proteins were phylogenetically compared. CCD8b was identified as a new class of cleavage dioxygenases that may play a specialized role in apocarotenoid biogenesis. A simple PCR assay was developed to measure *CCD1* gene copy number which is known to vary in maize. Using a panel of maize inbred lines varying in carotenoid content, linear regression analysis revealed a statistically significant negative correlation between copy number of *CCD1* and carotenoid content, an effect likely mediated through the resulting elevated levels of endosperm *CCD1* transcripts in high copy number lines. The PCR assay adds to a growing toolbox for metabolic engineering of maize endosperm carotenoids. This new tool can be used to select maize lines that are less likely to promote endosperm carotenoid degradation, thus predicting optimal results in metabolic engineering of endosperm provitamin A and/or nonprovitamin A carotenoids.

Introduction

In nature, carotenoids have diverse functions in both plants and animals. In plants, algae and cyanobacteria, carotenoids serve as structural and accessory pigments within the light harvesting complex to mediate photosynthesis and photoprotection. Carotenoids are also enzymatically cleaved to produce apocarotenoids, such as strigolactones, abscisic acid (ABA)[1] and other volatile compounds that contribute to the aroma of fruits and flowers [reviewed in 1–3]. Apocarotenoids play numerous physiological roles, including control of plant architecture, dormancy and stress responses and signals attracting beneficial mycorrhizal fungi that aid in nutrient uptake and promote plant growth. These same signals are utilized by harmful parasitic plants (e.g. *Striga*) that compete for plant resources [reviewed in 4].

The primary route in the formation of biologically active apocarotenoids is oxidative cleavage of carotenoids mediated by carotenoid dioxygenases [5,6]. Two types of carotenoid dioxygenases have been identified in plants, 9-cis carotenoid cleavage dioxygenases (NCEDs) and carotenoid cleavage dioxygenases (CCDs) (Fig. 1). These enzymes were identified by analyzing *viviparous* (*vp*) mutants of maize, which led to cloning of maize *NCED1* (*Vp14*) [7]. NCED enzymes cleave the 11,12 (11′,12′) double bond of 9-cis-violaxanthin or 9-cis-neoxanthin, catalyzing the first step in ABA biosynthesis [7,8]. In contrast, CCD enzymes do not share cleavage specificity with NCED enzymes. Some CCD enzymes recognize specific carotenoid or apocarotenoid substrates while others show promiscuity in choice of substrate as evidenced in heterologous systems [reviewed in 6,9–11]. CCD4 enzymes cleave β-carotene and/or 8′-apo-β-caroten-8-al, although there is no consistent CCD4 substrate among taxa, except for regioselectivity of cleavage at the 9,10 (9′,10′) positions [11,12]. In comparison, maize CCD1 has been shown *in vitro* to cleave a wide range of substrates (e.g. lycopene, β-carotene, zeaxanthin, etc.) and like other CCD1 enzymes, it is not regioselective in cleavage site [9,10,13]. CCD7 cleaves β-carotene asymmetrically, producing one β-ionone and C_{27} 10′-apo-β-carotenal; the latter product can be further cleaved by CCD8 generating C_{18} 13′-apo-β-carotenal [14] or CCD1 generating another β-ionone and one apo-10,10′-carotendial [13].

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[1] *Abbreviations used:* ABA, abscisic acid; CCDs, carotenoid cleavage dioxygenases; ESTs, expressed sequence tags; CT, threshold cycle; DAP, days after pollination.

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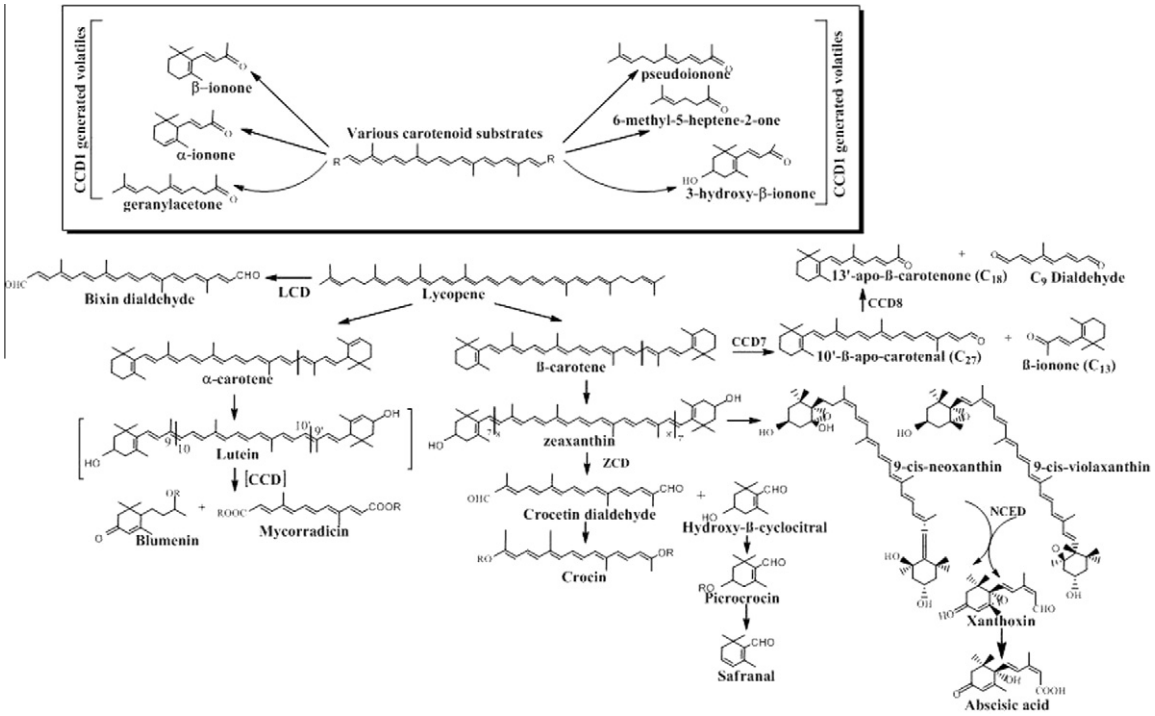

Fig. 1. Carotenoid cleavage dioxygenase activities in various species. CCD1 is promiscuous in the choice of substrate, thus producing an array of volatiles as shown inside the box [47]. Zeaxanthin cleavage dioxygenase, ZCD (*Crocus sativus*) [50]; lycopene cleavage dioxygenase, LCD (*Bixa orellana*) [5]. Formation of abscisic acid is mediated by 9-cis-epoxy carotenoid dioxygenases (NCED) [8]. CCD activity in mycorrhizal maize roots leads to formation of apocarotenoids mycorradicin and blumenin [22,51]; the hypothetical lutein substrate is indicated in square parentheses. CCD7 and CCD8 participate sequentially as shown [5].

Plant CCD enzymes generate various phytohormones and aroma compounds, although the cleavage pathways in plants are poorly understood. For example, *CCD1* expression causes the notable emission of diverse volatiles characteristic of fruit and flowers [15,16]. From studies in Arabidopsis, peas and rice, CCD7 and CCD8 were shown to be involved in generating the branching inhibition strigolactone hormones formed in roots [17–20]. These hormones signal symbiotic mycorrhizal fungal hyphae to branch and associate with plant roots or can be transported to aerial parts of the plant to inhibit branching or tillering of the plant, an important agronomic trait in the grasses [21].

Carotenoid dioxygenase genes have been identified in various plant species [22]. In the complete genome of *Arabidopsis*, nine genes similar to *VP14* were identified and characterized with regard to their tissue specific expression pattern and predicted subcellular localization [23–25]. Five genes encoding NCEDs are involved in ABA biosynthesis, while the remaining four genes encode CCDs.

Less is known about carotenoid cleavage enzymes in crops of agronomic importance. This enzyme class may potentially influence nutritional content (e.g. provitamin A carotenoids) and plant yield. The Poaceae includes the major food crops, for which carotenoid content is a target for improvement [reviewed in 26,27]. Efforts to engineer carotenoid accumulation in the grasses involve control of factors that influence net carotenoid accumulation, a balance of synthesis and degradation. Recent studies have shed light on the timing of expression and control points affecting pathway flux for carotenoid biosynthesis and accumulation in endosperm of maize [27–36]. Although there are some studies on a few individual cleavage enzymes, carotenoid degradation in the grasses is not well understood. This deficiency in knowledge will undoubtedly stymie attempts to breed high levels of carotenoids. Therefore this study focused on identifying and characterizing the entire carotenoid dioxygenase gene family in maize, rice, and sorghum.

Materials and methods

Sequence analysis and chromosome map localization

Rice genes (www.gramene.org) were used as a query to identify orthologs from *Zea mays* (www.tigr.org; www.plantgdb.org) and to decipher gene families. The genomic sequence, obtained from the respective BAC clones (Table 1), was used to deduce the full length cDNA sequence. The exon–intron boundaries were confirmed through selected ESTs, where available. Sequence analysis was performed using Vector NTI Suite 9.0 (Invitrogen, Carlsbad, CA). Translation of expressed sequence tags (ESTs) was used to distinguish gene paralogs. Chromosomal positions of genes in the *Z. mays* B73 inbred line were localized either by utilizing tools available at WebAGCoL package (http://www.agcol.arizona.edu/software/webagcol/) [37] or Maize GDB. Prediction of chloroplast targeting site was made using the ChloroP software (http://www.cbs.dtu.dk/services/ChloroP/) [38] and pSORT (http://wolfpsort.org/). Phytozome (http://www.phytozome.org) was used to retrieve sequences from *Sorghum bicolor* and all nonflowering plant and algal sequences. Phylogenetic analysis was performed using MEGA2 [39]. Deduced open reading frames and predicted proteins are presented in Tables S1 and S2, respectively.

Quantitative RT-PCR

Plants and tissues of different maize inbreds in a core subset of a maize germplasm collection were collected as described [29]. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen Sciences, Maryland), and DNase I-treated (Invitrogen, Carlsbad, CA) prior to first strand cDNA synthesis using oligo (dT) as a primer and SuperscriptTM III RT (Invitrogen, Carlsbad, CA). One microliters of 50 μM oligo $(dT)_{20}$ and 1 μl of 10 mM dNTP mix were mixed with 8 μl of DNase I treated total RNA (~1 μg) and incubated at 65 °C for