Characterization of Carotenoid Biosynthetic Pathway Using Viviparous Mutant Embryos in Maize(*Zea mays* L.)

Byung Moo Lee[1] and Nam Chon Paek[1,2]
[1]Dept. of Soil and Crop Sciences, Texas A&M Univ., College Station, TX 77843, USA.
[2]Kumho Life & Environmental Science Laboratory, Kwangju 506-712, Korea.

ABSTRACT

Carotenoid compounds in embryos of wild-type(WT) and viviparous mutants of maize(*Zea mays* L.) were analyzed using high performance liquid chromatography(HPLC) with a photodiode array detector. Zeaxanthin accumulates in WT embryos as the major carotenoid. Phytoene accumulates in *vp2* and *vp5*, phytofluene in *w3*, and ζ-carotene in the *vp9* mutant embryos. This indicates that the *vp2* and *vp5* mutants impair phytoene desaturase from 15-*cis*-phytoene to 15-*cis*-phytofluene. The *w3* mutant has neither an isomerase from 15-*cis*-phytofluene to all-*trans*-phytofluene nor phytofluene desaturase from phytofluene to ζ-carotene. The *vp9* mutant does not have the ζ-carotene desaturase from ζ-carotene to lycopene. Our analysis shows that the terminal carotenoid, γ-carotene(β,Ψ-carotene), accumulates in the vp7 mutant embryos. The ε-carotene(ε,ε-carotene), a product of δ-carotene(ε,Ψ-carotene) in some plants, however, has not been found in maize embryos. The *vp7* mutant impairs a cyclization step from γ-carotene to both β-carotene and α-carotene. We suggest that monocyclic γ-carotene is the sole precursor of both bicyclic β-carotene(β,β-carotene) and α-carotene(β,ε-carotene) in maize.

Key words: maize embryo, carotenoids, viviparous mutants, biosynthetic pathway

INTRODUCTION

Carotenoids are the most diverse and widespread pigments found in nature. Carotenoids are responsible for most of the yellow to red colors in flowers and fruits. These pigments are constituents of the chloroplast, where they act as light-harvesting pigments, as well as being involved in the electron transfer reactions. The synthesis of carotenoids occurs in the plastids of plants. The biosynthetic pathway of carotenoids is a branch of the well-known isoprenoid pathway originating from mevalonic acid(Fig. 1). As shown in Fig. 1, 15-*cis*-phytoene, 15-*cis* and all-*trans*-phytofluene are formed sequentially, and ζ-carotene, neurosporene and lycopene have the all-trans configuration in higher plants. Therefore, four dehydrogenation and one isomerization steps are needed in the desaturation pathway sequence from phytoene to lycopene, and two

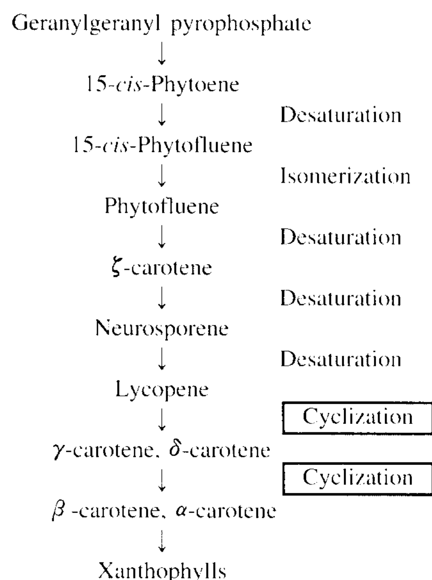

Fig. 1. Proposed pathway for the stepwise desaturation of phytoene into lycopene and cyclization from lycopene.

cyclization steps from lycopene to xanthophyll are required in higher plants(Bramley, 1985). The phytoene desaturase in *Arabidopsis*, soybean, tomato and pepper(Bartley et al., 1994) and the β and ε cyclases in *Arabidopsis*(Cunningham et al., 1996) have been cloned.

All of the maize mutants that are known to affect carotenoid synthesis have the common characteristic of producing viviparous kernels. These mutants are all recessive, and most of them have striking pleiotropic effects that facilitated their early discovery(Coe et al., 1988; Robertson, 1955 and 1975). Vivipary results from a series of anomalous metabolic events that arise from defects in a regulatory system during seed development. Vivipary is similar, but not identical, to germination(Paek et al., 1998).

With some modifications of Robertson's original classification(Robertson, 1955), the viviparous mutants in maize have been classified into three classes(Table 1), based on their primary functions and genetic lesion sites (Smith et al., 1989; Smith and Neuffer, 1992). Class I mutants affect a regulatory gene that requires ABA as a co-factor. *Viviparous-1(Vp1)* is the only gene identified so far in this class. The *vp1* mutant kernels have normal carotenoids (Fong et al., 1983) and normal level of ABA (Smith et al., 1978). Class II mutants(*vp2, vp5, vp7, vp9, w3, y3,* and *y9*) are carotenoid-deficient and cause lesions at various sites in the pathway between phytoene and xanthophylls(oxygenated carotenoids). The kernels in these mutants have different colors because carotenoid intermediates accumulate at the block in the biosynthetic pathway. These mutants are also ABA-deficient since each

Table 1. Class characteristics of viviparous mutants when homozygous recessive.

Pleiotropic Effect	Class I	Class II	Class III
Vp1 mRNA	No	Yes	Yes
Aleurone color†	Colorless	Purple	Purple
Kernel color	Yellow	White, Pink, Pale yellow	Yellow
Seedling color	Green	Albino	Green
Carotenoid level	Normal	Blocked	Normal
ABA level	Normal	Reduced	Reduced
ABA response	No	Yes	Yes

†when maize kernel has all of dominant alleles for anthocyanin synthesis.

mutation prevents the synthesis of the xanthophyll precursor of ABA. Class II mutants, except *y3* and *y9*, produce albino seedlings and eventually die(Robertson, 1975) due to photooxidation of chlorophyll in the absence of carotenoids(Krinsky, 1971, 1979). The *y3* mutant is temperature sensitive, and produces green/albino sectorial plants, while the *y9* mutant is leaky, and produces pale green seedlings(Smith et al., 1989). Class III mutants(*vp8, vp10,* and *vp14*) are only ABA-deficient. They interrupt ABA synthesis between the xanthophyll precursor and ABA. These mutants have normal carotenoids and produce green seedlings.

The purpose of this experiment is to identify the metabolites in the unique carotenoid biosynthetic pathway by analyzing carotenoid profiles of class II mutant embryos in maize. The most direct way to investigate each step of carotenoid biosynthesis is to use mutants that are completely blocked at a certain step in the sequential biosynthetic pathway, and analyze the intermediate metabolites accumulated in the mutants. This could be accomplished relatively easily if null mutants were available for every step of the pathway.

MATERIALS AND METHODS

Plant Materials

TX5855 is a standard inbred. All of mutant alleles were transferred into the TX5855 background by conventional backcrossing. Due to the lethality of homozygous mutants, normal seeds from self-pollinated ears segregating with the mutants were planted. Plants were self-pollinated, and segregating ears of self-pollinated heterozygous plants were harvested at 20 days after pollination. Harvested kernels were frozen in liquid nitrogen and stored at -70°C until analyzed. Both mutant and WT embryos from individual ears were isolated to analyze.

Chromatographic Equipment

A Waters Associates model 600E quaternary solvent delivery system(HPLC) equipped with the Waters model 996 photodiode array detector and Waters model 717 autosampler with 200μL injection loop were used. The data were processed and obtained using Millennium software.

Table 2. Absorbance maxima of carotenes extracted from embryos of maize viviparous mutants compared with published values.

Carotene	Retention Time(min)	Extracted λ_{max}(nm)			Published λ_{max}(nm)			Reference‡
Lycopene	30.19	450.9	475.2	504.4	449	474	506	1
γ-Carotene	33.23	436.3	460.6	489.8	440	462	492	2
ζ-Carotene	36.36	383.2	402.5	426.6	381	402	426	1
Phytofluene	39.01	336.6	351.0	368.7	334	350	368	2
Phytoene	41.74		288.9			286		2

‡ 1: Rouseff et al., 1992; 2: Khachik et al., 1992.

Extraction and Analysis of Carotenoids

Frozen embryos were ground with a mortar and pestle in hexane containing butylated hydroxytoluene(BHT)(0.01%). Each extract was filtered, and the filtrate was dried under nitrogen gas. The residue was resuspended in 200 μL of hexane containing BHT. Samples were chromatographed by HPLC on a 4.6×250mm 5μm Microsorb C_{18} column(Rainin Instrument Co.). An isocratic mixture of acetonitrile(85%), methanol(10%), dichloromethane(2.5%) and hexane(2.5%) at time 0 was followed by a linear gradient beginning at 10min and completed at 40min. The final gradient mixture of acetonitrile(45%), methanol(10%), dichloromethane(22.5%) and hexane(22.5%) was followed at 45 min. The column flow rate was 0.7mL min^{-1}. At the end of the gradient, the column was re-equilibrated under the initial isocratic conditions for 15min at a flow rate of 2mL min^{-1} and finally for 5min at 0.7mL min^{-1}. Elution of the various compounds was monitored by their absorbance at four different wavelengths(290, 350, 400 and 460nm). β-Apo-8'-carotenal(Fluka Chemical Corp.) was used as the internal standard. This system was useful for identification of various carotenes, but it did not separate lutein-5,6-epoxide from antheraxanthin or lutein from zeaxanthin(Khachik et al., 1992).

Identification of Carotenoids

Carotenoids were identified initially by comparing their retention times and spectra with both published UV-visible spectra and those of standards (Braumann and Grimme, 1981). Quantitation of carotenoids was based on calibration curves of β-carotene, and corrections were made for differences in extinction coefficients.

RESULTS AND DISCUSSION

Identification of Carotenoids

Because of the lability of carotenoids, it was important to proceed rapidly with extraction, identification and measurement of carotenoids. All procedures were carried out in the dark or dim light because the carotenoid compounds are very sensitive to light. Exposure to extremes of pH and high temperatures was avoided. Extraction was completed within 10 min, and a single reverse-phase HPLC step was sufficient to separate carotenoids of interest.

The absorbance spectra of carotenoids recorded using the photodiode array detector were compared with published values in Table 2. The spectra have been normalized for comparison purposes. With the exception of phytoene, all the carotenoids showed the characteristic three-fingered spectra. Both all-*trans* and 15-*cis* isomers of phytoene and phytofluene have the same absorbance spectra in this HPLC system(Khachik et al., 1989; Rouseff et al., 1992). The absorbance maxima obtained agreed favorably with the published values. The relative retention times of carotenoids on reverse-phase HPLC were also in agreement with reported relative retention times in similar systems(Khachik et al., 1989).

Carotene Levels in Class II Mutants

The carotenoid contents of the class II viviparous mutant embryos in maize are in Table 3. WT embryos accumulate zeaxanthin as the major carotenoid. Carotenogenesis is blocked at various steps in the class II mutants. Both *vp2* and *vp5* embryos accumulate phytoene, *w3* phytofluene, and *vp9* ζ-carotene, in agreement with the findings reported

Table 3. Carotenoid levels in extracts of embryos of maize viviparous mutants.

Genotype	Phytoene	Phytofluene	ζ-Carotene	Lycopene	γ-Carotene
			μg/g frozen wt		
vp2	50.56				
vp5	35.50				
w3	91.85	0.13			
vp9	12.69	4.18	27.51		
vp7	0.19	0.01	ND[a]	47.08	3.37

[a]ND: Not detected.

previously(Fong et al., 1983; Neill et al., 1986). The conversions of 15-*cis*-phytoene into 15-*cis*-phytofluene, 15-*cis*-phytofluene into all-*trans*-phytofluene, and all-*trans*-phytofluene into ζ-carotene have been reported(Bramley, 1985). This suggests that at least two desaturases and one isomerase are required for those conversions. The *vp2* and *vp5* mutants impair phytoene desaturase from 15-*cis*-phytoene to 15-*cis*-phytofluene. The *w3* mutant impairs either an isomerase between 15-*cis*-phytofluene and all-*trans*-phytofluene or phytofluene desaturase from phytofluene to ζ-carotene. The *vp9* mutant impairs ζ-carotene desaturase from ζ-carotene to lycopene. Additionally, we identified the terminal carotenoid accumulated in the *vp7* mutant embryo was γ-carotene. Both γ-carotene(β, Ψ-carotene) and δ-carotene(ε, Ψ-carotene) have been suggested to be precursors of β-carotene(β, β-carotene) and α-carotene(β, ε-carotene) in maize, respectively(Fong et al., 1983). However, ε-carotene(ε, ε-carotene), a product of δ-carotene, has not been found in maize(Bramley, 1985) and *Arabidopsis*(Cunnigham et al., 1996). Therefore, we suggest with previous reports(Bramley, 1985; Cunnigham et al., 1996) that monocyclic γ-carotene is the sole precursor of both bicyclic β-carotene(β, β-carotene) and α-carotene(β, ε-carotene). We conclude that the *vp7* mutation impairs a cyclization step from γ-carotene to β-carotene and α-carotene.

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