Production of high-value compounds: carotenoids and vitamin E

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The unraveling in recent years of the pathways and genes that are responsible for biosynthesis of vitamins and carotenoids in plants has provided new molecular tools that can be utilized to genetically modify the content and composition of these compounds *in vivo*. There have been recent examples of successful manipulation of carotenoid composition and vitamin E activity in plants using gene-transfer technology.

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Abbreviations

γ-TMT	γ-tocopherol methyl transferase
GGDP	geranylgeranyl diphosphate
HGA	homogentisic acid
HPPDase	p-hydroxyphenylpyruvic acid dioxygenase
IDP	isopentenyl diphosphate
PDS	phytoene desaturase
PSY	phytoene synthase

Introduction

Plant breeding has been focusing over the years mainly on increasing crop productivity by pursuing higher yields and better adaptation to biotic and abiotic stresses. Improving nutrient composition of plant foods, however, is no less important. There are still vast populations, mainly in the developing world, whose diet lacks essential nutrients, such as vitamins and minerals. For example, it was estimated that over 124 million children worldwide are vitamin A deficient, and that improved vitamin A nutrition alone could prevent 1.3–2.5 million deaths among late infancy and preschool-age children that occur each year in the developing countries [1,2]. Modern breeding tools of gene transformation open up the possibility for metabolic engineering targeted at improving nutritional quality of plants. To manipulate complex metabolisms it is necessary to unravel the biochemical pathways and understand the regulation underlying metabolic fluxes. This paper examines the progress made in understanding the biosynthesis and regulation of carotenoids (vitamin A) and tocopherols (vitamin E) and describes attempts to manipulate these pathways.

Carotenoids (vitamin A)

Carotenoids are 40-carbon isoprenoids, which consist of eight isoprene units. Carotenes are hydrocarbons that are either linear or cyclized at one or both ends of the molecule. Oxygenated derivatives of carotenes are called xanthophylls. The polyene chain, consisting of conjugated double bonds, is responsible for their characteristic colors and their photochemical properties [3]. In plants, carotenoids are essential for photosynthesis where they serve as accessory light-harvesting pigments and as photoprotectants that quench tissue-damaging free radicals such as singlet oxygen species [4,5]. They also function in pigmentation of flowers and fruits.

All carotenoid species that contain a β-ring can be converted to retinol and are thus precursors for vitamin A. Although this is the major importance of carotenoids in human nutrition, there are other health benefits that are attributed to carotenoids, even to those that lack provitamine A activity, such as lycopene [6,7]. These are thought to be associated with the activity of carotenoids as antioxidants. Manipulating carotenoid biosynthesis in plants has additional agronomic significance because they furnish distinct colors to flowers and fruits. Carotenoids are also added to fish and poultry feed and used as colorants in the cosmetics and food industries.

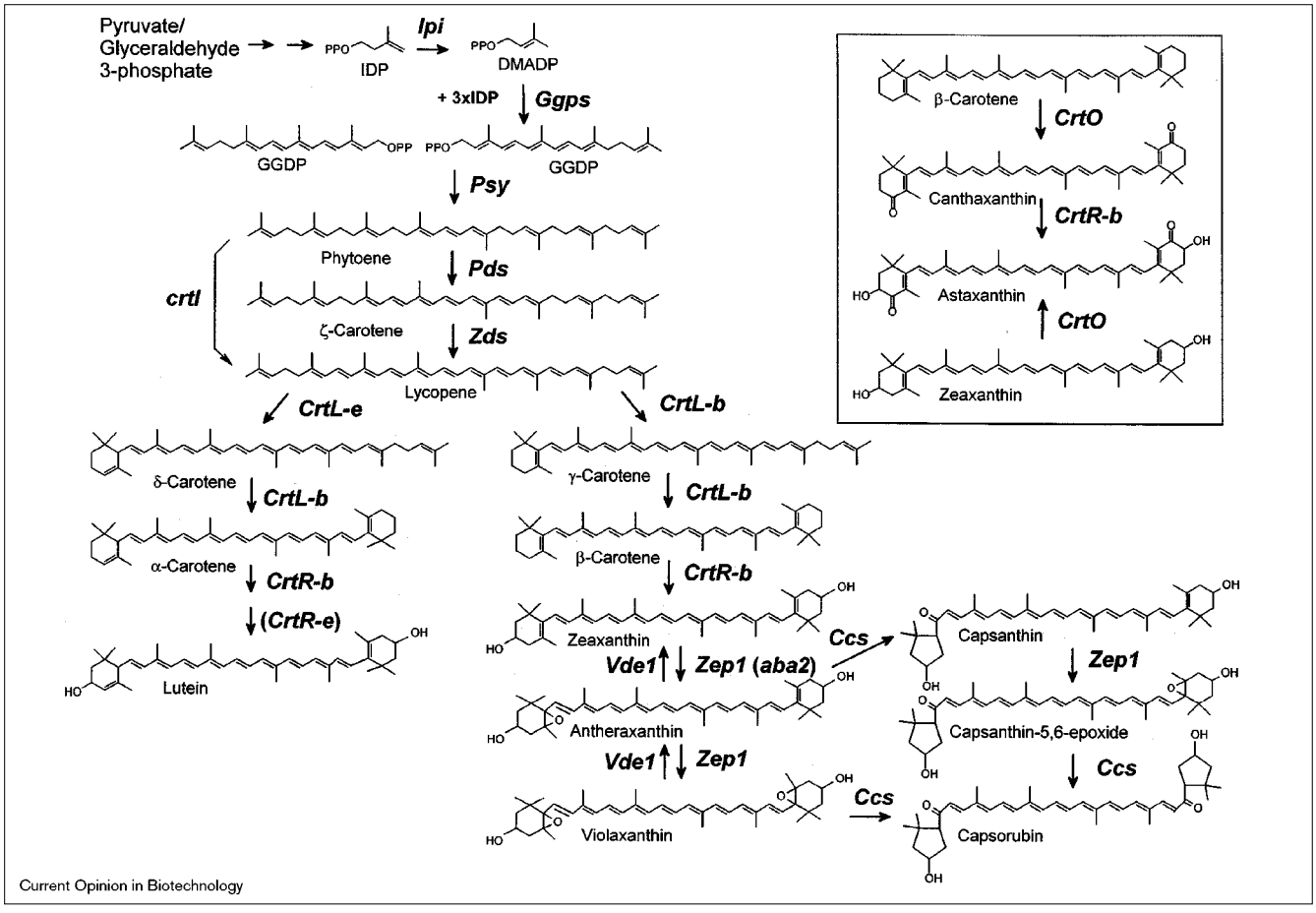
Carotenoid biosynthesis

The carotenoid biosynthesis pathway was postulated over three decades ago by standard biochemical analyses using labeled precursors, specific inhibitors and characterization of mutants [8]. Lack of *in-vitro* assays for most of the enzymes, however, hindered a more detailed elucidation of this pathway. In recent years, genes encoding nearly all of the enzymes in the carotenoid biosynthesis pathway have been cloned from bacteria, fungi and plants using various genetic techniques (reviewed in [9–13]; Figure 1). Their characterization has provided new information on both the enzyme activities and the regulation of the pathway.

Carotenoids are synthesized within the plastids from the central isoprenoid pathway, which also serves the synthesis of other important compounds, such as terpenes, squalene, gibberellins, sterols and phytol. All isoprenoids are built from the 5-carbon compound isopentenyl diphosphate (IDP). The major source of IDP in plastids is from pyruvate and glyceraldehyde-3-phosphate, not from acetyl-CoA via 3-hydroxy-3-methylglutaryl-CoA and mevalonic acid as previously suggested [14]. IDP is isomerized to dimethylallyl diphosphate by the enzyme IDP isomerase (encoded by the gene *Ipi*). The activity of this enzyme was found to be a rate-limiting step in yeast and bacteria and, therefore, could also have the same property in plants [15,16]. Sequential addition of three IDP molecules to dimethylallyl diphosphate is catalyzed by a single enzyme, geranylgeranyl diphosphate (GGDP) synthase, and produces the 20-carbon molecule GGDP. Five isoforms of GGDP synthase, each encoded by a different gene (*Ggps*), exist in *Arabidopsis*; it is not clear yet whether all of them take part in GGDP synthesis.

The first committed step to the carotenoid pathway is the head to head condensation of two GGDP molecules to

Figure 1



The carotenoid biosynthesis pathway in plants. The astaxanthin biosynthesis pathway in the alga *H. pluvialis* is boxed. Enzymes are named according to the designation of their genes. *Ccs*, capsanthin-capsorubin synthase; *crtI*, phytoene desaturase (bacterial type); *CrtL-b*, lycopene β-cyclase; *CrtL-e*, lycopene ε-cyclase; *CrtO*, β-C-4-oxygenase (ketolase); *CrtR-b*, β-ring hydroxylase, *CrtR-e*, ε-ring hydroxylase; DMADP, dimethylallyl diphosphate; *Ggps*, geranylgeranyl diphosphate synthase; IDP, isopentenyl diphosphate; *Ipi*, IDP isomerase; *Pds*, phytoene desaturase; *Psy*, phytoene synthase; *Vde*, violaxanthin deepoxidase; *Zds*, ζ-carotene desaturase; Zep (*aba2*), zeaxanthin epoxidase. Genes for all the enzymes except *CrtR-e* have been cloned.

produce phytoene, catalyzed by the enzyme phytoene synthase (PSY; Figure 1). Introduction of four double bonds converts phytoene to lycopene. This is carried out in plants by two enzymes, phytoene desaturase (PDS) and ζ-carotene desaturase, each catalyzes two symmetric dehydrogenation steps to yield ζ-carotene and lycopene, respectively. These enzymes bind FAD and use plastoquinone as an electron acceptor. The cyclization of lycopene is an important branching point in the pathway. One route leads to β-carotene and its derivative xanthophylls — zeaxanthin, violaxanthin and neoxanthin. The alternative pathway leads to carotenoids with one β-ring and one ε-ring, such as α-carotene and lutein, which is a major xanthophyll in the leaves of most higher plants. All carotenoid biosynthesis genes in plants and algae are nuclear encoded and their polypeptide products are imported to the plastids.

Carotenoid formation is a highly regulated process. Concentration and composition of leaf xanthophylls in chloroplasts are affected by light intensity and the accumulation of specific carotenoids in chromoplasts of fruits and flowers is developmentally regulated. In tomato, for example, total carotenoid concentration increases between 10- to 15-fold during fruit ripening, due mainly to a 500-fold increase in the concentration of lycopene [17]. It was established that expression of the genes *Ggps*, *Psy*, and *Pds* is upregulated during fruit development just prior to color appearance (reviewed in [11,12]), whereas the expression of the genes for the two lycopene cyclases, *CrtL*-b and *CrtL*-e, is abolished at this stage [18,19•]. Consequently, lycopene accumulates to high concentrations in the ripe fruit. Developmental regulation of transcription of the above genes is the principal control mechanism of carotenoid biosynthesis also in chromoplasts of bell pepper [20].

Metabolic engineering of carotenoids

Cloning the genes for the carotenoid biosynthesis enzymes has opened the way for genetic manipulation of this biosyn-

thetic pathway in plants. Carotenoid biosynthesis was introduced into *Escherichia coli* (reviewed in [21]) and yeast [22•], indicating the possibilities for genetic manipulations of the pathway *in vivo* in other systems. As PSY catalyzes the first committed step in the pathway, it has been a preferred target for gene manipulation. Attempts to elevate carotenoid levels by up-regulating *Psy* expression, however, have so far been disappointing. Constitutive expression of the cDNA of *Psy* in transgenic tomato plants has led to dwarfism due to redirecting GGDP from the gibberellin pathway into carotenoids, which reduced the levels of gibberellin hormones in the plants [23]. Fruits in these plants produced lycopene earlier in development but the final levels of lycopene in the ripe fruits were lower than in the control [24]. Other attempts to highly express *Psy* have caused co-suppression of the indigenous *Psy* leading to inhibition of carotenoid biosynthesis. Similar inhibition of carotenoid biosynthesis was obtained in tobacco plants that over-expressed *Pds* following viral infection [25]. Inhibition of carotenoid biosynthesis in tomato fruits was obtained through anti-sense silencing of *Psy* [26,27].

A significant accomplishment, however, was achieved in transgenic expression of the daffodil cDNA of *Psy* in rice. Under the regulation of an endosperm-specific promoter, *Psy* expression resulted in synthesis and accumulation to a low level of phytoene in this tissue [28••]. This is the first step in an attempt to install β-carotene biosynthesis in rice seeds aimed at increasing its provitamin A value.

Expression in tobacco of the *Erwinia crtI* gene for phytoene desaturase, which catalyzes four desaturations of phytoene to give lycopene, has conferred tolerance to the herbicide norflurazon [29]. This herbicide inhibits PDS but not CRTI. Expression of CRTI caused minor changes in xanthophyll composition in the leaves in favor of lutein at the expense of violaxanthin. A modest increase in total carotenoids was observed in ripe fruits of transgenic tomatoes expressing CRTI, but the profile was altered so that β-carotene was elevated by 30% and lycopene was slightly reduced [24].

Using an RNA viral vector, the capsanthin-capsorubin synthase cDNA was highly expressed in tobacco plants [30]. Leaves from transfected plants developed an orange phenotype and accumulated high levels of capsanthin (up to 36% of total carotenoids). In pepper, these red xanthophylls accumulate to high concentrations within chromoplasts in specific lipoprotein fibrils. When synthesized in chloroplasts, however, they caused thylakoid membrane distortion, reduction of grana stacking and a decrease of the other major leaf xanthophylls.

The gene *crtO*, which encodes β-C-4-oxygenase, a key enzyme in ketocarotenoids synthesis, was cloned from the alga *Haematococcus pluvialis* [31]. Expression of the algal *CrtO* gene in the *Synechococcus* PCC7942, which normally synthesizes β-carotene and zeaxanthin, enabled the cyanobacterial cells to produce astaxanthin along with other ketocarotenoids [32]. This result confirmed that *crtO* can function in conjunction with the intrinsic β-carotene hydroxylase that exist in all plants and algae to produce novel carotenoid species. Transformed tobacco plants that expressed *CrtO* in a regulated manner accumulated high concentration of ketocarotenoids, including astaxanthin, in the chromoplasts of the nectary tissue in the flowers, changing their color from yellow to red (J Hirschberg, unpublished data). It is important to note that the astaxanthin that was produced in the transgenic plants and cyanobacteria had the same chirality (3*S*3′*S*) as the natural astaxanthin that is found in marine organisms. In contrast, the synthetic astaxanthin that is currently used as fish feed is a mixture of stereoisomers of which 75% have the unnatural chiral structure. These results demonstrate the prospect to genetically engineer carotenoid biosynthesis towards the production of commercially valuable compounds in plants.

Tocopherols (vitamin E)

Tocopherols (collectively known as vitamin E) are a class of lipid-soluble antioxidants, which are essential ingredients in human nutrition [33]. Epidemiological evidence has indicated that vitamin E supplementation results in decreased risk for cardiovascular disease and cancer, aids in immune function, and prevents or slows a number of degenerative diseases associated with aging, such as cataracts, arthritis, and disorders of the nervous system, caused by cumulative damage to tissues mediated by reactive oxygen species. As tocopherols are synthesized only by photosynthetic organisms, the major source for vitamin E in our food is from plants. Tocopherols exist at different concentrations in various plant species and also vary between tissues. Whereas leaves of most common plants contain low levels of tocopherols (10–50 μg/g fresh weight) they can accumulate to high concentrations (500–2000 μg/g) in seeds [34,35]. Seed oils are the major source of naturally derived dietary α-tocopherol.

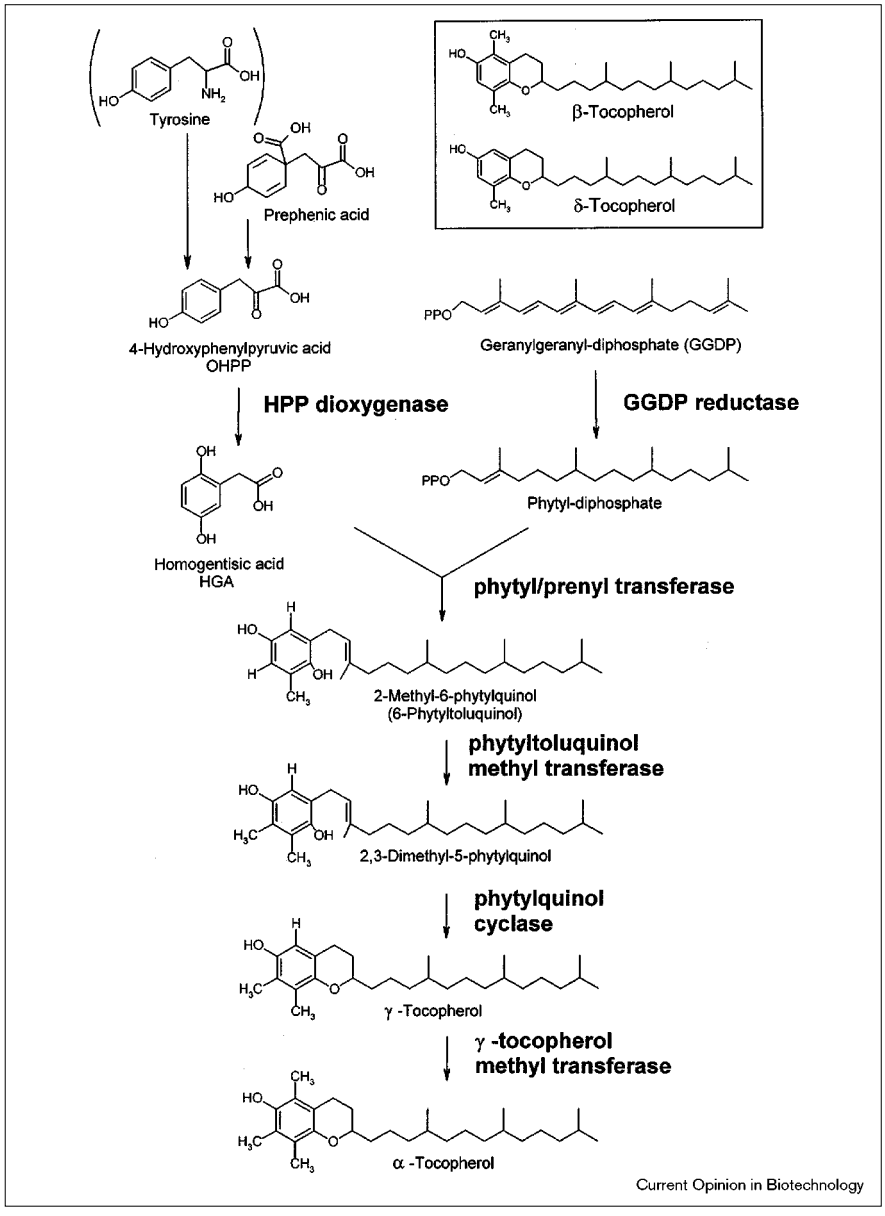
Tocopherols are amphipathic molecules consisting of a polar head group and a hydrophobic tail. Because of this biochemical property, they are associated with membrane lipids or accumulate in lipid storage cellular structures. The four major tocopherols, α, β, γ and δ differ only in the number and position of methyl substituents on the aromatic head group (Figure 2). The *in vivo* antioxidant activity of α-tocopherol is higher than the other species, about tenfold higher than that of its immediate biosynthetic precursor γ-tocopherol [36]. The nutritional value of tocopherols as depicted in vitamin E activity is determined, therefore, by the concentration of α-tocopherol [37]. Natural α-tocopherol occurs as a single (*R,R,R*)-α-tocopherol isomer, whereas synthetic α-tocopherol (which is mainly used in vitamin E supplements) is a racemic mixture of eight different stereoisomers. Most of these isomers are less efficacious than the (*R,R,R*) isomer [33].

Biosynthesis of tocopherols

Tocopherols are synthesized from precursors of two pathways: the isoprenoid pathway, which provides the

Figure 2

The tocopherol biosynthesis pathway in plants. HPP, p-hydroxyphenylpyruvic acid.



hydrophobic tail, and that of homogentisic acid formation, which provides the head group (Figure 2). The biosynthetic pathway in plants was elucidated three decades ago using classical biochemical studies [38,39]. Many details remained obscured, however, due to lack of molecular description. Recently, significant progress was made at the molecular level regarding the synthesis and accumulation of tocopherols in plant tissues. Mutation analysis, gene cloning and novel genomic technologies contributed new information on key steps in the pathway and provided the necessary tools for biotechnological approaches towards manipulating vitamin E levels in plants.

The biosynthetic pathway of tocopherols is depicted in Figure 2. Homogentisic acid (HGA) supplies the head group for both tocopherols and quinones. HGA is produced from p-hydroxyphenylpyruvic acid in a complex enzymatic reaction that is catalyzed by the cytosolic enzyme p-hydroxyphenylpyruvic acid dioxygenase (HPPDase), which is encoded in *Arabidopsis* by a single locus *PDS1*. A mutation in *PDS1* indicated that this step is essential for both tocopherol and plastoquinone biosynthesis [40]. The cDNAs encoding HPPDase were cloned from various plants [41,42•]. A phytyl sidechain is added to HGA simultaneously with a decarboxylation reaction by phytyl/prenyl transferase to form the first tocopherol intermediate, 2-methyl-6-phytylplastoquinol. This plastid-located enzyme, which is encoded by a single locus (*PDS2*) in *Arabidopsis*, also catalyzes the prenylation reaction that adds solanyl pyrophosphate to HGA in the plastoquinone biosynthesis pathway [40]. Phytyl pyrophos-

phate is produced from GGDP by a step-wise reduction catalyzed by geranylgeranyl reductase. The cDNA that encodes this enzyme has been cloned [43]. GGDP is produced from IDP in the central isoprenoid pathway by the enzyme GGDP synthase (see above).

The next step in α-tocopherol synthesis is methylation by phytyltoluquinol methyl transferase of 2-methyl-6-phytylplastoquinol at position 3 to yield 2,3-dimethyl-6 -phytylplastoquinol, which is followed by cyclization to yield d-7,8 dimethyltocol (γ-tocopherol). Finally, a second ring methylation at position 5 yields α-tocopherol [44] (Figure 2). It is still unclear how δ- and β-tocopherols are produced, but their synthesis proceeds from 2-methyl-6-phytylplastoquinol. The phytylquinol cyclization enzyme has been purified to homogeneity and biochemically characterized from *Anabaena variabilis* [45]. The γ-tocopherol methyl transferase has been purified from cyanobacteria and higher plants [46,47] and recently cloned from cyanobacteria and *Arabidopsis* [48••].

Genetic manipulation of α-tocopherol

Deciphering the biosynthetic pathway of α-tocopherol and the availability of cloned genes for key enzymes have made metabolic engineering of the pathway possible. One can predict rate limiting steps whose up-regulation could increase the flux through the pathway to reach relatively higher amounts of tocopherols in a given plant tissue. Availability of HGA and the phytol sidechain are potentially important in this respect. Manipulating the enzymes HPPDase, phytyl/prenyl transferase and GGDP reductase could, therefore, have a significant effect on the levels of tocopherol production. As in other central biochemical pathways, however, the fluxes are also influenced by other inter-connecting pathways, in the case of tocopherols these are the isoprenoids and quinones, so that the net effect might be hampered.

Another manipulation that is now possible is aimed at qualitative changes in tocopherol composition. An exquisite and very significant example of metabolic engineering in this direction has recently been reported by Shintani and DellaPenna [48] who cloned and characterized the cDNA of γ-tocopherol methyl transferase (γ-TMT) from *Arabidopsis*. The full-length cDNA of γ-TMT was over-expressed in transgenic *Arabidopsis* seeds using the seed-specific carrot DC3 promoter. *Arabidopsis* seeds contain ~370 ng total tocopherols per milligram that are composed mainly (97%) of γ-tocopherol. In the transgenic seeds, 85–95% of the total tocopherol pool was α-tocopherol, representing an 80-fold increase in α-tocopherol levels compared with the wild-type control. Interestingly, total seed tocopherol levels were not altered in these plants, indicating that either there is no stringent feed-back regulation of the pathway in seeds by γ-tocopherol or that α-tocopherol functions in the same mechanism as γ-tocopherol. This qualitative change in tocopherol composition reflects a ninefold increase in total vitamin E activity of the seed oil due the different vitamin E potency of α- versus γ-tocopherol. This is the first example of increasing a vitamin level in a plant tissue by molecular manipulation. Similar increases in vitamin E activity as a result of γ-TMT over-expression can be envisioned for commercially important oils.

Conclusions

Current understanding of the carotenoids and tocopherols biosynthesis in plants enables the genetic manipulation of these pathways. Modifying existing pathways has achieved alteration in composition of tocopherols and diversion of carotenoids to a novel high-value species. Attempts to increase the concentration of these compounds, however, have yielded so far only limited results.

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