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# Current strategies for vitamin E biofortification of crops

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Vitamin E refers to four tocopherols and four tocotrienols that are exclusively synthesized by photosynthetic organisms. While α-tocopherol is the most potent vitamin E compound, it is not the main form consumed since the composition of most major crops is dominated by γ-tocopherol. Nutritional studies show that populations of developed countries do not consume enough vitamin E and that a large proportion of individuals exhibit plasma α-tocopherol deficiency. Following the identification of vitamin E biosynthetic genes, several strategies including metabolic engineering, classic breeding and mutation breeding, have been undertaken to improve the vitamin E content of crops. In addition to providing crops in which vitamin E content is enhanced, these studies are revealing the bottlenecks limiting its biosynthesis.

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## Introduction

Vitamin E encompasses eight organic compounds with a chromanol ring substituted with one to three methyl groups that are collectively called tocochromanols. Vitamin E isoforms with a saturated prenyl side chain are called tocopherols, while the ones with an unsaturated prenyl side chain are named tocotrienols (Figure 1a & b). Tocopherol and tocotrienol forms (α-, β-, γ-, and δ-) differ by the number and position of methyl substituents on the chromanol ring. The vitamin E activity of each form differs greatly due to the different affinities between specific tocochromanols and the liver α-tocopherol transfer protein, which preferentially binds α-tocopherol [1] (Table 1). Other tocochromanols such as plastochromanol-8 (PC-8; Figure 1c) and tocomonoenols (Figure 1d) have been identified in edible plants regularly consumed by humans [2,3]. The vitamin E activity of these latter forms is unknown yet.

Vitamin E was originally identified as a nutritional factor essential for animal reproduction [4]. Since, it has been shown that vitamin E is a powerful lipid antioxidant that protects cell membranes from the damaging effects induced by free radicals [5,6]. In addition, several tocochromanol isoforms show beneficial roles in delaying brain aging [7], reducing the risk of developing Alzheimer's disease [7], or inhibiting lung cancer [8]. Tocopherols are found in virtually all photosynthetic plants, notably in vegetable oil and nuts, whereas tocotrienols are mainly encountered in monocot seeds. Despite its wide availability, nutritional surveys clearly demonstrate that a large portion of Western populations do not meet the vitamin E recommended dietary allowance [9,10,11••]. While the long-term effects on health of chronic vitamin E deficiency are not definitively established for humans, the oxidative stress associated with vitamin E deficiency strongly advocates for improving its quality and quantity in the human diet.

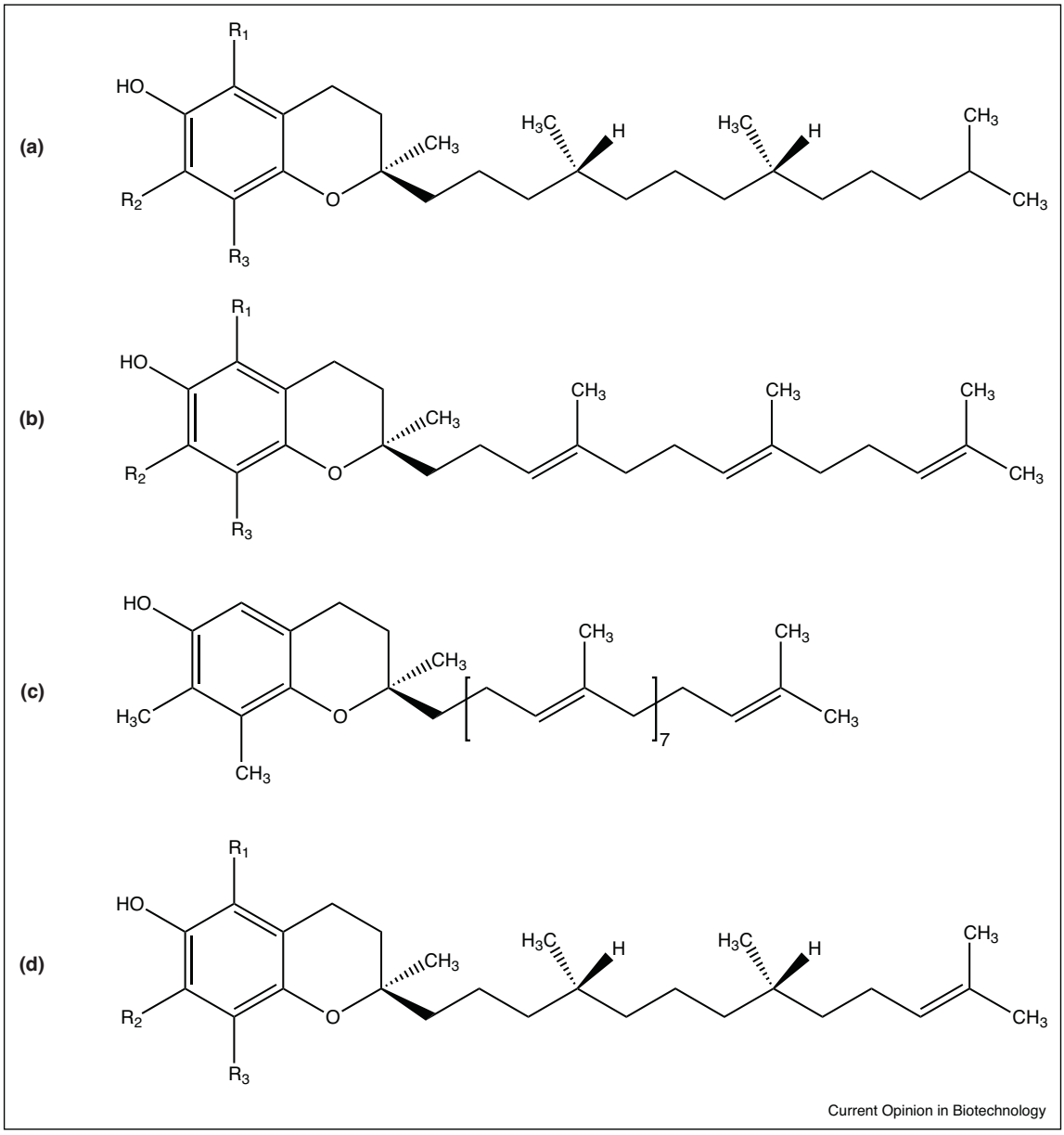
### Update on the tocochromanol biosynthetic pathway

Strategies implemented to enhance vitamin E content in crops derive from our current knowledge of tocochromanol biosynthesis, which has been recently reviewed [12,13]. This section will briefly summarize tocochromanol metabolism and introduce vitamin E biosynthetic genes recently identified (Figure 2).

Tocochromanols are amphipathic molecules composed of a polar chromanol ring built around the phenolic compound homogentisic acid (HGA), and a hydrophobic isoprenoid side chain. HGA is produced from 4-hydroxyphenylpyruvate (HPP) by a *p*-hydroxyphenylpyruvate dioxygenase (HPPD; Figure 2a). In plants, HPP derives from tyrosine degradation that notably involves a specific tyrosine aminotransferase [14]. In contrast, cyanobacteria produce HPP directly from chorismate and/or prephenate with a bifunctional chorismate mutase/prephenate dehydrogenase named TyrA [15,16].

The prenyl side chains of prenylquinols and tocochromanols all derive from geranylgeranyl pyrophosphate (GGPP) produced by the plastidial methyl erythritol phosphate (MEP) pathway (Figure 2b). Geranylgeranyl pyrophosphate synthase (GGPPS) 11 was recently shown to catalyze the biosynthesis of most plastid GGPP-derived isoprenoids, including tocochromanols [17••]. For tocotrienol synthesis, GGPP is directly condensed with HGA. For solanesyl derivatives such as plastoquinol-

**Figure 1**




Tocochromanol structures. Chemical structures of tocopherols **(a)**, tocotrienols **(b)**, plastochromanol-8 **(c)**, and tocomonoenols **(d)**. $R_1$, $R_2$, $R_3 = CH_3$, α-tocochromanol; $R_1$, $R_3 = CH_3$, $R_2 = H$, β-tocochromanol; $R_2$, $R_3 = CH_3$, $R_1 = H$, γ-tocochromanol; $R_3 = CH_3$, $R_1$, $R_2 = H$, δ-tocochromanol.

9 (PQ-9) and PC-8, GGPP is converted into solanesyl pyrophosphate (SPP) by solanesyl pyrophosphate synthases [18]. Tocopherol synthesis requires the reduction of GGPP into phytyl pyrophosphate (PPP) by a geranylgeranyl reductase (GGR). *In vitro*, GGR reduces both free GGPP and geranylgeranylated chlorophyll *a* (Figure 2c). Recently, the light-harvesting-like proteins LIL3:1 and LIL3:2 were shown to be involved in tocochromanol metabolism through their interaction and stabilization of GGR proteins in the chloroplast membrane [19]. In Arabidopsis seeds and leaves, tocopherol synthesis mostly depends on the phytol kinase VTE5 and the phytyl phosphate kinase VTE6 that sequentially phosphorylate phytol into PPP [20,21••]. In senescent leaves, the phytol used for tocopherol synthesis originates mostly from chlorophyll hydrolysis [21••]. In Arabidopsis

**Table 1**

**Vitamin E activity of natural and synthetic tocochromanols. The biological activity of each tocochromanol form, given in IU/mg and compared to the activity of α-tocopherol, has been calculated from the rat fetal resorption assay. Briefly, vitamin E-depleted virgin females were mated with normal males. Pregnant females were subsequently fed with different doses of specific vitamin E isomers during 21 days after which they were sacrificed. The vitamin E biological activity was determined by counting the number of living, dead, and resorbed fetuses. One international unit (IU) corresponds to the vitamin E activity of 1 mg of the synthetic *all-rac*-α-tocopheryl acetate**

| Tocochromanol | Activity (IU/mg) | Activity (%) |
|---|---|---|
| α-Tocopherol | 1.49 | 100 |
| β-Tocopherol | 0.75 | 50 |
| γ-Tocopherol | 0.15 | 10 |
| δ-Tocopherol | 0.05 | 3 |
| α-Tocotrienol | 0.45–0.75 | 30–50 |
| β-Tocotrienol | 0.08 | 5 |
| γ-Tocotrienol | Below detection | Below detection |
| δ-Tocotrienol | Below detection | Below detection |
| *all-rac*-α-Tocopheryl acetate (synthetic) | 1 | 67 |
| *RRR*-α-Tocopheryl acetate (synthetic) | 1.36 | 91 |

seeds, chlorophyll degradation and recycling contributes at least 60% to tocopherol synthesis [20]. In contrast, the origin of phytol used for tocopherol synthesis in healthy leaves is still an open question [22••].

The committed step of tocochromanol biosynthesis is the condensation of HGA with an isoprenoid side chain mediated by various prenyltransferases. Tocotrienol synthesis is initiated by a homogentisate geranylgeranyltransferase (HGGT), PC-8 synthesis by a homogentisate solanesyltransferase (HST), and tocopherol synthesis by a homogentisate phytyltransferase (HPT; Figure 2e). The resulting methylprenylquinols are methylated by methyltransferase (MT) to form dimethylprenylquinols. Both methyl- and dimethyl-geranylgeranyl- and phytyl-quinols are cyclized by tocopherol cyclase (TC) to form δ-tocochromanols and γ-tocochromanols, respectively. These isoforms are further methylated by the γ-tocopherol methyl transferase (*γ-TMT*) to form β-tocochromanols and α-tocochromanols, respectively. For solanesyl derivatives, cyclization of PQ-9 by TC produces PC-8 that is not further methylated in wild-type plants.

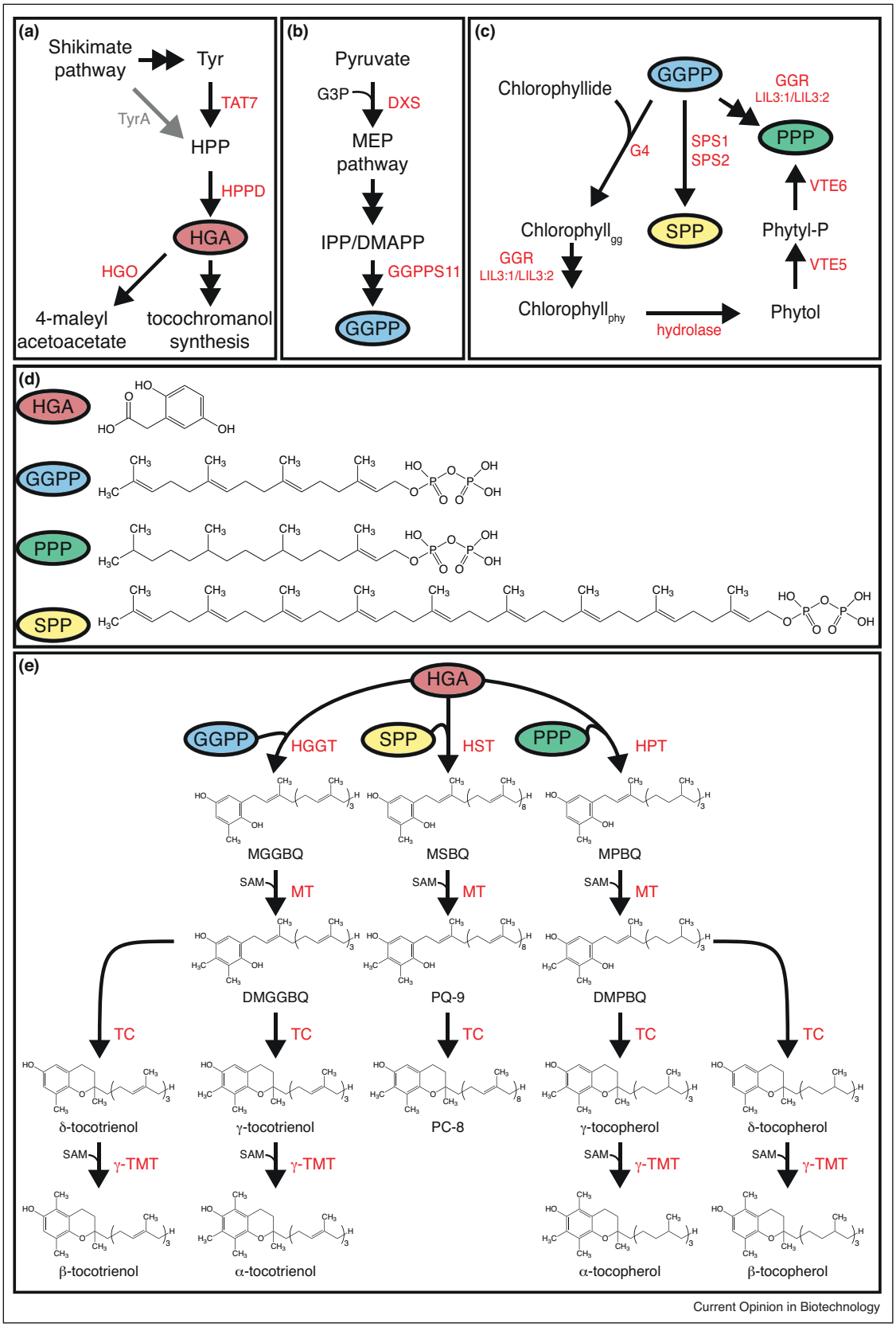
### Vitamin E biofortification through improving plant tocopherol composition

The first strategy that was assessed to enhance vitamin E activity in plants consisted in improving plant tocochromanol composition by converting preexisting tocopherols into forms exhibiting higher biological potency [23]. Indeed, seeds of the most abundantly consumed oilseed crops (*e.g.*, soybean, rapeseed, cotton, and oil palm) accumulate primarily γ-tocopherol, a form that has 10% of the vitamin E activity of α-tocopherol (Table 1). Since γ-tocopherol is the direct precursor of α-tocopherol, its conversion into α-tocopherol by *γ-TMT* overexpression should greatly enhance the vitamin E activity in a tissue. This strategy was originally attempted in Arabidopsis in which the *γ-TMT* gene was introduced under a seed-specific promoter [23]. While γ-tocopherol dominated the tocopherol composition of wild-type seeds (>95%), α-tocopherol represented up to 95% of seed tocopherols in the best transgenic event [23]. Subsequently, the successful conversion of γ-tocopherol into α-tocopherol via *γ-TMT* overexpression has been reported in many other plants including soybean [24–27], shiso [28], lettuce [29], mustard [30], maize [31], and tobacco [32]. Because of the higher biological activity of α-tocopherol, most *γ-TMT* overexpressing crops exhibited 5–10 times higher vitamin E activity than untransformed plants. In species accumulating δ-tocopherol, which has 3% of the vitamin E activity of α-tocopherol, *γ-TMT* overexpression also enhanced its conversion into β-tocopherol, which at 50% of the vitamin E activity of α-tocopherol is 16.6 times more potent [23–27,30] (Figure 2 and Table 1). Because the gain in vitamin E activity is very significant and no adverse effects on growth and fertility have been reported in *γ-TMT* overexpressing plants, this strategy is today among the most effective for vitamin E biofortification of crops.

In addition to transgenic approaches, α-tocopherol enrichment can also be achieved by traditional breeding using natural high α-tocopherol alleles identified in crop germplasms by QTL studies. While seeds of most soybean varieties contain low α-tocopherol amounts (<10% of the tocopherol pool), three varieties having up to 53% of α-tocopherol were identified in soybean germplasm [33]. QTL analysis showed that high α-tocopherol content correlated with higher expression of *γ-TMT3*, a soybean gene encoding a polypeptide that exhibits 81.8% similarity with the Arabidopsis *γ-TMT* protein [34]. Comparison of *γ-TMT3* promoter sequences between high and standard α-tocopherol varieties identified conserved polymorphisms within the promoter regions of the high α-tocopherol varieties that might be responsible for the higher *γ-TMT3* promoter activity. This hypothesis was confirmed in transgenic Arabidopsis plants expressing the *GUS* reporter gene fused to *γ-TMT3* promoters originating from standard or high α-tocopherol varieties [34]. Thus, soybean germplasm carries monogenic alleles that increase by at least fivefold the α-tocopherol content in seeds and could be introgressed into standard varieties to improve their vitamin E content.

High α-tocopherol cultivars have been identified in other crops including rice [35•], maize [36,37], and rapeseed [38]. Collectively, these high α-tocopherol natural variants represent promising alternatives to transgenic crops, notably for countries in which the production and/or marketing of plant GMOs are currently banned.

**Figure 2**



### Tocopherol biosynthetic precursor availability regulates vitamin E accumulation

In order to identify the biosynthetic steps limiting tocochromanol accumulation in plants, tocopherol production was investigated in cell cultures fed with metabolic intermediates [15,39,40]. While these studies concluded that both HGA and phytol had the greatest effects on vitamin E biosynthesis, their conclusions differ on which compound is the most limiting. In safflower cell cultures, phytol feeding significantly increased tocopherol production after 3 days of incubation, while HGA supplementation had no effect [39]. After fourteen days of treatment, HGA feeding increased tocopherol content by 3.3 times, while phytol supplementation increased tocopherol content by 18.4 times [39]. These data indicate that the availability of both tocopherol precursors restricts vitamin E biosynthesis, with phytol being the most limiting. In contrast, in sunflower suspension cells, HGA feeding stimulated tocopherol accumulation (~30%), while phytol supplementation had no effect [40]. Finally, in soybean suspension cultures, tocopherol content increased twofold in response to either HGA or phytol feeding, and fivefold when both precursors were added simultaneously [15]. These studies collectively showed that the availability of tocochromanol biosynthetic precursor(s) significantly limits vitamin E accumulation in plants and suggest that enhancing the metabolic pathway(s) producing them *in planta* might improve vitamin E synthesis.

### Vitamin E biofortification through metabolic engineering of tocopherol precursors

The second strategy aiming at improving crop vitamin E activity involves increasing the global production of tocochromanols. This was attempted by overexpressing tocopherol core biosynthetic genes (*VTE*), and biosynthetic genes producing the aromatic head and/or the isoprenoid side chain of prenylquinols.

#### *Transgenic approaches targeting vitamin E core biosynthetic genes* (VTE)

The committed step of the tocochromanol pathway is catalyzed by prenyltransferases that condense HGA with either PPP (tocopherols) or GGPP (tocotrienols). Attempts to increase tocochromanols by overexpressing prenyltransferases in plants led to contrasting results depending on the selected gene, the plant organ or species, and the study. Overexpression of *HPT* genes in canola and soybean did not significantly increase seed tocopherol content [15]. In Arabidopsis, *HPT* overexpression increased seed tocopherols from 40% to 100% depending on the study, indicating that this activity is at least partially limiting in Arabidopsis seeds [15,41,42]. In leaves, *HPT* overexpression induced a 3.6 fold and 4.4 fold increase of tocopherol content in tomato and Arabidopsis, respectively [42,43]. Together, these indicate that, while the HPT activity is not the major bottleneck in seed tocopherol metabolism, it clearly restricts tocopherol synthesis in leaves.

Overexpression of *HGGT* genes increased tocochromanol content up to 5 times in tobacco leaves, up to 15 times in Arabidopsis leaves, and 7–18 times in maize kernels [44–46]. In agreement with the preference of HGGT for GGPP, newly synthesized tocochromanols were exclusively tocotrienols. In Arabidopsis and tobacco leaves overexpressing *HGGT*, tocopherol content was not altered despite the massive accumulation of tocotrienols [44,46]. Since tocopherol and tocotrienol biosynthetic pathways both share HGA as a common precursor, these data indicate that Arabidopsis and tobacco leaves can sustain tocochromanol synthesis up to 15 and 5 times the WT tocochromanol levels, respectively, without HGA being limiting. In transgenic maize kernels the situation is slightly different. While maize *HGGT* overexpressors containing 7 times higher tocotrienol content showed unchanged tocopherol amounts, lines accumulating 18 times more tocotrienols had an 18% reduction in tocopherols [44,45]. These indicate that in maize, HGA is not limiting up to a certain enhancement of tocochromanol metabolism, but does become limiting at higher levels. In addition, these data also show that HGGT activity is clearly limiting tocotrienol biosynthesis in maize kernels.

#### *Transgenic approaches targeting HGA availability*

Since HGA feeding improved tocopherol content in some studies, transgenic approaches aiming at increasing

**(Figure 2 Legend)** Biosynthetic pathways involved in tocochromanol biosynthesis. Biosynthetic pathways forming the tocochromanol precursors homogentisic acid (HGA, **a**), geranylgeranyl pyrophosphate (GGPP, **b**), phytyl pyrophosphate (PPP) and solanesyl pyrophosphate (SPP, **c**), as well as prenylquinols and tocochromanols **(e)**. The chemical structures of HGA, GGPP, PPP and SPP are shown **(d)**. Compound abbreviations: DMAPP, dimethylallyl pyrophosphate; DMGGBQ: 2,3-dimethyl-6-geranylgeranyl-1,4-benzoquinol; DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinol; G3P, D-glyceraldehyde-3-phosphate; HPP, 4-hydroxyphenylpyruvate; IPP, isopentenyl pyrophosphate; MGGBQ, 2-methyl-6-geranylgeranyl-1,4-benzoquinol; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; MSBQ, 2-methyl-6-solanesyl-1,4-benzoquinol; PC-8, plastochromanol-8; PQ-9, plastoquinol-9; SAM: *S*-adenosyl-methionine; Tyr, tyrosine. Biosynthetic enzyme abbreviations: DXS, 1-deoxy-D-xylulose-5-phosphate synthase; HGGT, homogentisic acid geranylgeranyl transferase; HGO, homogentisic acid dioxygenase; HPPD, *p*-hydroxyphenylpyruvate dioxygenase; HPT, homogentisic acid phytyl transferase (VTE2); HST, homogentisic acid solanesyl transferase; G4, chlorophyll synthase; GGPPS11, geranylgeranyl pyrophosphate synthase 11; GGR, geranylgeranyl reductase; *γ-TMT*, γ-tocopherol methyltransferase (VTE4); LIL3, light harvesting-like protein 3; MT, methyltransferase (VTE3); SPS, solanesyl pyrophosphate synthase; TAT7, tyrosine aminotransferase 7; TC, tocopherol cyclase (VTE1); TyrA, bifunctional chorismate mutase/prephenate dehydrogenase; VTE5, phytol kinase; VTE6, phytyl phosphate kinase. The direct synthesis of HPP from chorismate and/or prephenate via the prokaryotic TyrA enzyme (grey arrow) exists in cyanobacteria but not in higher plants.

synthesis of this precursor *in planta* have been assessed. Initial attempts to increase HGA synthesis consisted in overexpressing *HPPD* genes (Figure 2a). While *HPPD* overexpression slightly increased tocopherol and tocotrienol contents in seeds of transgenic tobacco [47], it did not substantially increase tocochromanols in tobacco or Arabidopsis leaves, or in Arabidopsis and soybean seeds [15,47–50]. In contrast, in the cyanobacterium *Synechocystis*, *HPPD* overexpression resulted in a sevenfold increase in tocopherols [15]. The contrasting results of *HPPD* overexpression in higher plants and photosynthetic bacteria likely results from the difference in HPP biosynthesis between these organisms. While (cyano)bacteria and yeast produce HPP directly from chorismate and/or prephenate via a bifunctional chorismate mutase/prephenate dehydrogenase, its biosynthesis in higher plants requires several enzymatic reactions involving tyrosine as an intermediate [15,16]. Since tyrosine levels are tightly regulated in plant cells via feedback inhibition by tyrosine, plant tocochromanol biosynthesis may be restricted by tyrosine availability, whereas photosynthetic bacteria escape this limitation [13,16]. This hypothesis was assessed in transgenic plants co-expressing *HPPD* genes together with bacterial or yeast prephenate dehydrogenases (*TyrA* and *Tyr1*, respectively). In Arabidopsis and tobacco leaves, overexpression of *HPPD* alone did not alter tocopherol contents but its co-expression with *TyrA* or *Tyr1* induced a twofold and 10-fold increase in tocochromanols, respectively [48,50]. This indicates that HGA availability limits tocochromanol synthesis in leaves of these two species. In seeds, co-expression of *HPPD* and *TyrA* increased tocochromanol amounts in Arabidopsis (+80%), canola (+140%), and soybean (+160%), indicating that HGA availability also partially restricts tocochromanol biosynthesis in seeds [15]. Importantly, newly synthesized tocochromanols in plants co-expressing *HPPD* and *TyrA* genes were mostly tocotrienols, including in species or tissues that usually do not accumulate them [15,48,50]. This indicates that HGA availability restricts tocotrienol synthesis but is not sufficient *per se* to enhance tocopherol synthesis.

In Arabidopsis and soybean seeds, co-expression of *HPPD* and *TyrA* increased free HGA content 60-fold and 800-fold, respectively [15]. Since newly synthesized HGA is only partially converted into tocochromanols, these data collectively show that HGA availability is one of several limitations that restrict tocochromanol biosynthesis in plants. This was further demonstrated by overexpressing additional vitamin E biosynthetic gene(s) into high-HGA transgenic plants [15]. For instance, *HPT* overexpression in high-HGA Arabidopsis and soybean further increased tocochromanol synthesis [15]. The highest tocochromanol increase (15-fold) was reported for high-HGA soybeans in which both *HPT* and *GGR* were further overexpressed [15]. In these latter examples, newly produced tocochromanols were mostly tocotrienols confirming that seed tocopherol biosynthesis is not limited in the first place by HGA availability.

A novel mechanism limiting HGA availability and tocochromanol metabolism has been recently identified in the soybean MO12 mutant [51••]. This deletion mutant notably lacks *HGO1*, a gene encoding an HGA dioxygenase that catalyzes the degradation of HGA into 4-maleylacetoacetate (Figure 2a). Since free HGA levels partially control tocochromanol biosynthesis, its degradation by HGA dioxygenase might potentially limit vitamin E biosynthesis. Indeed, MO12 mutant seeds contained 30 times higher HGA amounts and twice as much tocochromanols [51••]. As for high-HGA transgenic plants, tocotrienol content was strongly enhanced in MO12 mutant seeds, while tocopherols were unchanged. Collectively these data demonstrate that soybean HGA dioxygenase restricts tocochromanol synthesis in seeds by degrading HGA. In addition, it further demonstrates that increasing only HGA availability is not sufficient to boost tocopherol synthesis in seeds.

### *Transgenic approaches targeting the isoprenoid side chain synthesis*

Several transgenic approaches targeting PPP synthesis have been assessed in plants. It has been shown that Arabidopsis lines overexpressing the phytol kinase gene (*VTE5*) accumulated wild-type tocopherol amounts in transgenic seeds [20]. In addition, Arabidopsis transgenic lines overexpressing the phytyl phosphate kinase gene (*VTE6*) accumulated at best 15% more γ-tocopherol in seeds [21••]. Together, these data show that the conversion of phytol into PPP is not a significant bottleneck in vitamin E synthesis, at least in Arabidopsis seeds.

Because phytol has been shown to be limiting, these data suggest that pathway(s) involved in GGPP synthesis, and/or chlorophyll degradation, and/or the reduction of GGPP into PPP might regulate isoprenoid availability in plants, and therefore tocochromanol synthesis. The first hypothesis has been partially demonstrated in transgenic Arabidopsis in which overexpression of the *1-deoxy-D-xylulose 5-phosphate synthase* (*DXS*), the first enzyme of the MEP pathway (Figure 2b), doubled tocopherol content in transgenic Arabidopsis seedlings [52]. It was later shown that *DXS* overexpression in mature Arabidopsis leaves did not significantly alter isoprenoid contents, indicating that DXS activity might be limiting only in young tissues [53].

A second mechanism controlling PPP availability has been identified in tobacco in which the constitutive expression of *CHL P*, a gene encoding a GGR, induced a sixfold increase of tocopherols in leaves and a threefold increase in seeds (Patent US 6,624,342 B1). To date, these amounts are the highest tocopherol increases ever reported in both seeds and leaves, indicating that reduction of GGPP into PPP is likely the most limiting step of

tocopherol biosynthesis in plants, more so than HPT activity or HGA availability. This conclusion is in agreement with the results of the high-HGA transgenic plants overexpressing both *HPPD* and *TyrA* or *Tyr1* that accumulated mainly unsaturated tocochromanols (tocotrienols) rather than saturated ones (tocopherols) [15,48,50]. However, these data still do not explain why high-HGA transgenic soybeans expressing GGR overaccumulated tocotrienols instead of tocopherols [15].

## Conclusions & future challenges

During the last two decades, very significant progress has been made on our understanding of vitamin E biosynthesis in photosynthetic organisms and a total of 19 different genes that impact or are required for tocochromanol synthesis have been identified in plants (Figure 2). Several strategies have been undertaken to improve vitamin E content in crops. To date, the overexpression of the *γ-TMT* gene is by far the most potent method available. Regardless of the host or the transgene origin, *γ-TMT* overexpressors and naturally elevated expression in high α-tocopherol accessions exhibit the highest vitamin E increases thus far reported in plants.

The numerous metabolic engineering studies published so far greatly improved our understanding of both tocotrienol and tocopherol metabolisms in plants. In species naturally producing tocotrienols such as monocot seed, tocotrienol content is strongly restricted by *HGGT* expression. In contrast, tocopherol content is not strongly affected by *HPT* overexpression in seeds. In addition, the massive accumulation of tocotrienols rather than tocopherols in transgenic plants overaccumulating free HGA or overexpressing *HGGT* demonstrated that tocopherol synthesis is not primarily regulated by the availability of free HGA. Since phytyl pyrophosphate mostly comes from the degradation and recycling of chlorophylls, at least in senescent leaves and seeds accumulating chlorophylls such as Arabidopsis, it suggests that identifying the genes and regulatory mechanism(s) involved in chlorophyll turnover will likely open up new horizons for vitamin E biofortification of crops. This new frontier might be challenging since chlorophylls are essential for plant survival and metabolism and their biosynthesis and turnover are both tightly regulated in plant tissues. This also questions the origin of phytol/phytyl pyrophosphate and tocopherol synthesis in species whose seeds do not produce chlorophylls.

## Acknowledgements

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- • of special interest
- •• of outstanding interest


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