https://doi.org/10.15407/frg2021.06.484
UDC 581.1

STRATEGIES FOR INCREASING ALPHA-TOCOPHEROL CONTENT IN PLANTS

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The main ways of obtaining α-tocopherol (α-T) is through chemical synthesis and the extraction of α-T from plant oils. A widely used synthetic form called *all-rac*-α-tocopherol consists of mixing eight stereoisomers with part of natural stereoisomer RRR-α-tocopherol only 12.5 %. Natural α-T is 1.5 times more active than synthetic forms, that is why the search for effective sources of natural α-T continues. Plant oils such as sunflower, maize, rapeseed and soybean are the main sources of natural commercial vitamin E with low activity due to low level of α-T content in seed oils. Much research has been done that shows stimulating of α-T production in plant cells by changing cultivation conditions, including light intensity, photoperiod, nitrogen level, temperature, and type of carbon nutrition etc. Stress conditions all stimulate the accumulation of antioxidants in the photosynthetic organisms, but can also restrict their normal growth rate. Gene engineering allows for the creation of plants with high α-T content by introducing the coding sequences (CDS) of significant genes of tocochromanol synthetic pathway into a nuclear genome of transgenic plants. cDNA with CDS of key enzymes of the α-T synthesis, such as homogentisate geranylgeranyl transferase (HGGT), tocopherol cyclase (TC), γ-tocopherol methyltransferase (γ-MTM) from rice, soybean, maize, carrot etc., are used to enhance the total tocochromanol content and improve tocopherols composition. Application of the combination of biotechnology techniques, genetic engineering and optimization of cultivation conditions, greatly stimulates the accumulation of α-T in photosynthetic organisms.

Key words: α-tocopherol, vitamin E, tocochromanol, biotechnology, transgenic plants.

Ways of increasing α-tocopherol content in plants. The activity of tocochromanols is defined as the activity of vitamin E. Humans and animals are unable to synthesize these lipophilic antioxidants crucial for their well-being. Therefore, the only way is to rely on photosynthetic microorganisms, plants and algae as sources of tocopherol. Intensive research are currently to find ways of improving production of biologically active vitamin E by photosynthetic microorganisms and plants [1, 2].

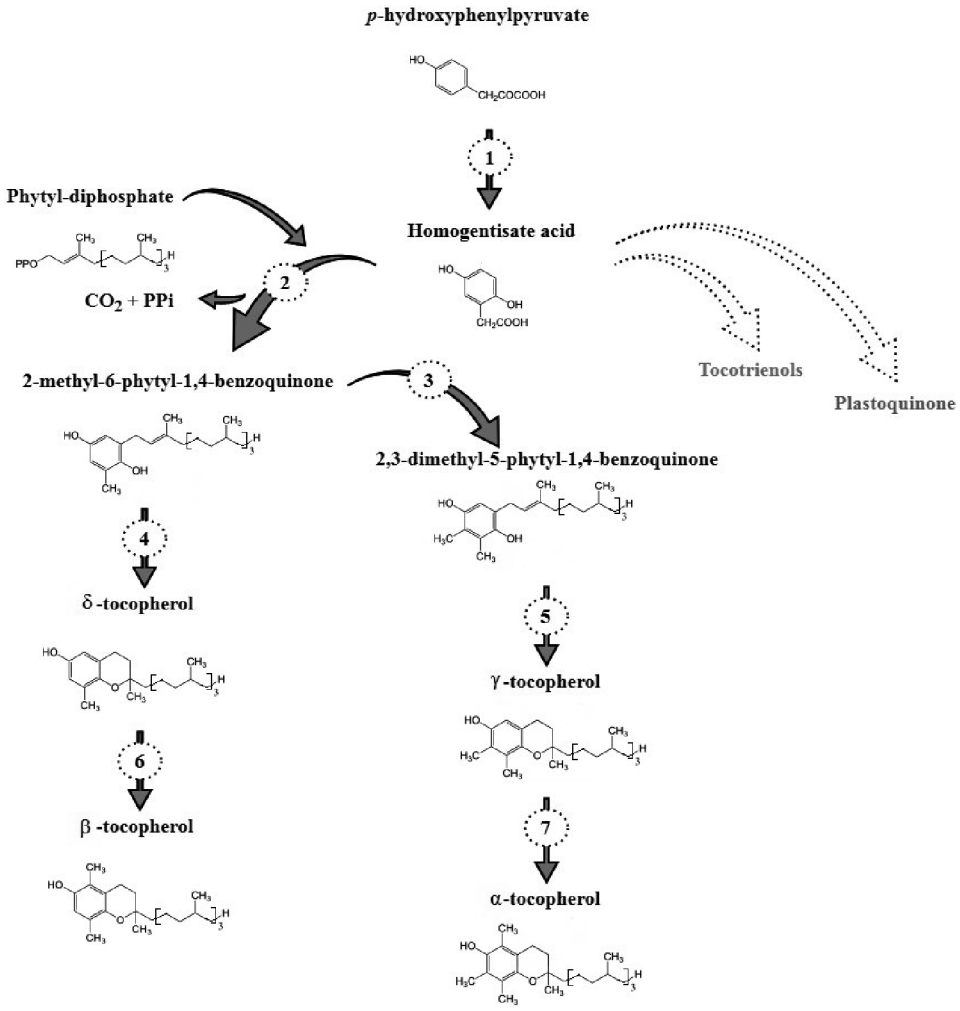
The problem researchers face with obtaining tocopherol from higher plants is the rather low level of these compounds in mentioned sources, especially of the most active isoform — α-tocopherol. Soybean oil is one

Citation: Mokrosnop V.M., Zolotareva E.K. Strategies for increasing alpha-tocopherol content in plants. Fisiol. rast. genet., 2021, 53, No. 6, pp. 484—500. https://doi.org/10.15407/frg2021.06.484

of the most consumed plant oils throughout the world by humans. It contains ~100 mg tocopherols in 100 g. However, the activity of vitamin E in this oil is low precisely because of the small content of α-tocopherol, which amounts to 10—20 %, whereas γ-tocopherol content is around 60—65 % from the total tocopherols, δ-tocopherol — 20—26 % and β-tocopherol — 2—5 % [3]. Selection is one of the biotechnological methods of acquiring plants with high content of active vitamin E, i.e. with an increased amount of α-tocopherol. The method involves the usage of specific molecular markers, which bind with the genes responsible for controlling the levels of α- and γ-tocopherol in plants, thereby enabling the selection of required alleles of genes for altering a ratio of α-/γ-isoforms. It leads to attaining the seed with a slightly increased content of α-T and γ-isoform as a dominant form. The activity of γ-tocopherol as vitamin E is three times less than α-T [4]. Application of transgenic methods can result in the significantly higher accumulation of α-T [5].

Genetic engineering helps to increase the level of active vitamin E in cultivated plants, especially in oil-plants. While aiming to achieve this goal, researchers were trying to alter the expression of structural genes responsible for encoding the major synthesis enzymes of the tocochromanols [6, 7]. There is already evidence of successful implementation of such strategies, which demonstrated quality improvement of transgenic plants as producers of vitamin E, compared to the compound levels in the wild type (WT) [8].

Engineering tocopherol biosynthetic pathway. The vitamin E biosynthetic pathway was elucidated in 1979, but the genes encoding the enzymes for most of the pathway steps have been identifed only in the last decade with using genetic and genomic-based methods [9, 10]. To date, all key enzymes in the pathway have been isolated and used to establish genetic manipulations of plants to increase the total tocopherol content and improve the composition of vitamin E [4, 11—13]. Genetic experiments conducted on model organisms (*Arabidopsis thaliana*, *Synechocystis* PCC6803) helped to identify genes and proteins, which take part in tocochromanol synthesis [8]. The following key enzymes were characterized: *p*-hydroxyphenylpyruvate dioxygenase (HPPD), homogentisate phytyltransferase (HPT), tocopherol cyclase (TC), 2-methyl-6-phytyl-1,4-benzoquinone methyltransferase (MPBQ MT), γ-tocopherol methyltransferase (γ-OIO) (Figure). Increasing and decreasing the rate of expression of single genes as well as groups of tocopherol biosynthesizing genes, it was possible to reveal the metabolic processes that influence tocopherol accumulation and improve the content of these compounds in plants [7]. The accumulation of the most active isoform of vitamin E in the cells of transgenic plants is achieved mainly in two ways: conversion of accumulated tocopherol into α-T or increase in concentration of all tocochromanol isoforms. Both strategies were successfully applied to *Arabidopsis*, rapeseed, and soybeans. The seed of these plants contains mainly γ- and δ-tocopherol due to the reduced activity of γ-TMT (*VTE4*) which methylate γ- and δ-tocopherol to, respectively, α- and β-tocopherol (see Figure). HPPD overexpression in *Arabidopsis* has been reported to enhance α-T levels by 1.37-fold in leaves and by 1.28-fold in roots compared to wild type [14]. In tobacco plants, overexpression of HPPD only slightly increased vitamin E



Tocopherol biosynthesis pathway in *A. thaliana* (adapted from Fritsche, Wang & Jung [10]). Numbers in circles refer to the enzymes and corresponding genes in Table.

Enzymes and genes of the tocopherol biosynthesis in A. thaliana corresponding to the reactions shown on Figure

Reaction	Enzyme	Gene	Substrate → Product	Increased ratio of tocopherol isoforms
1	*p*-Hydroxyphenyl-pyruvate dioxygenase (HPPD)	*PDS1*	HPP → HGA	Total
2	Homogentisate phytyltransferase (HPT)	*VTE2*	HGA+PDP → MPBQ	Total
3	2-Methyl-6-phytyl-1,4-benzoquinone methyltransferase (MPBQ MT)	*VTE3*	MPBQ → DMPBQ	(γ+α)/total
4, 5	Tocopherol cyclase (TC)	*VTE1*	MPBQ, DMPBQ → δ-tocopherol, γ-tocopherol	(β+α)/total
6, 7	γ-Tocopherol methyltransferase (γ-TMT)	*VTE4*	δ-Tocopherol, γ-tocopherol → β-tocopherol, α-tocopherol	α/γ and β/δ

content in seeds but did not increase vitamin E content in leaves [11]. HPPD catalyzes the formation of HGA, which in turn is involved not only in the synthesis of tocopherols, but also plastoquinone in plant cells, therefore no significant increase in the level of tocopherols upon activation of HPPD expression can be observed.

Overexpression of homogentisic acid phytyl transferase (*VTE2*/HPT) in *Arabidopsis* and tomato increased the tocopherol content in leaves by about 3.6- and 4.4-fold, respectively [10, 15]. Overexpression of the *VTE2* gene in *Arabidopsis* and soybean seeds increases the total level of tocopherol by 1.8 and 1.4 times, respectively [1]. A 5-fold increase in the concentration of all isoforms of tocopherols in leaves and 2-fold in *Arabidopsis* seeds was achieved by embedding the *VTE2* gene from *Synechocystis* sp. The highest content of vitamin E in *Arabidopsis* seeds compared to the wild type was observed in transgenic plants with united modification of three genes: *VTE2, HPPD and TyrA* encoding prephenate dehydrogenase — an enzyme involved in the shikimate pathway. However, overexpression of VTE2 in soybean and canola did not increase seed tocopherol content significantly [16].

The level of expression of other vitamin E biosynthetic pathway enzymes such as γ-tocopherol methyl transferase (*VTE4*), catalysed the final step for α-T production; tocopherol cyclase (*VTE1*) and 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase (*VTE3*), that catalyze penultimate steps in the α-T biosynthesis, also influences tocopherol content in plants. The accumulation of tocochromanols in the leaves and seeds of plants is also achieved by expression of homogentisine geranylgeranyl transferase (HGGT) [1, 6, 17].

Plant transformation, which is directed to an increase in the total content of tocopherols, is carried out by increasing the expression activity of *HPPD* and VTE2 genes, and the ratio of tocopherol isoforms is regulated by *VTE1, VTE3*, and *VTE4* genes (Tabl). An increase in the expression of the *HPPD* and *VTE2* genes does not affect the amount of the α-isoform fraction, which remains insignificant in the seeds. Overexpression of *VTE1* was shown to enhance α-T levels by 4-fold in tobacco plants compared to the wild type [18]. The content of α-T was 1.6—3.3-fold higher in sweet potato (*Ipomoea batatas* [L.] Lam) leaves overexpressing *VTE1* (*IbTC*) than in non-transgenic leaves. No significant difference was observed in the tocopherol content of storage roots between genetically modified and non-modified plants [19].

Overexpression of the *VTE3* and *VTE4* genes in *Arabidopsis* plants reduces the content of δ- and β- tocopherol isoforms with a proportional increase in γ- and α-isoforms, while the content of α-T reaches 97.1 % without changing the total amount of tocopherols [3, 20]. Transgenic *Arabidopsis* plants with joint overexpression of the *VTE2* and *VTE4* genes contained 4.5 times the total amount of tocopherols with a percentage of α-T 91.3 % [21]. Transformation of *Arabidopsis* plants, aimed at increasing the expression of *VTE4* in seeds using the seed-specific carrot DC3 promoter, led to the conversion of > 95 % of γ-tocopherols to α-isoform, and a small pool of δ-tocopherols to β-tocopherol without increasing the total content of tocochromanols. Thus, the activity of vitamin E accumu-

lated in *Arabidopsis* seeds increased by 9 times [22]. This type of genetic modification is in demand in commercially important oilseeds transformation, such as rapeseed, soybean, and corn, which have reduced amounts of active vitamin E. As a result of soybean gene transformation with embedding of the *VTE4* and *VTE3* genes from *A. thaliana*, the ratio of tocopherol isoforms changes with an increase in the fraction of α-T to 96—98 %, β-tocopherol to 2—4 %, and a decrease in γ- and δ-isoforms to less than 0.2 % [3]. When the *VTE4* gene isolated from *Perilla frutescens* was transformed into soybean and perilla, the α-T content increased in transgenic seeds by 10.4- and 26.6-fold, respectively, and the α-/γ-tocopherol ratio in transgenic lines increased from 0.12 to 280.0 and 0.05 to 3.22, respectively [13, 23]. In a recent study, Zhang et al. [24] showed that overexpression of corn *VTE4* (*ZmTMT)* increased the α-T content 4—5-fold in transgenic *Arabidopsis* and around 6.5-fold in transgenic maize kernels, and increased the α-/γ-tocopherol ratio to approximately 15 and 17, respectively [24].

In addition to traditional genetic engineering strategies new methods such as genome-wide association study (GWAS) and genome editing are developed in enhancing tocopherol accumulation in plant [21, 25]. GWAS is a relatively new way for scientists to identify genes or quantitative trait loci underlying complex traits in plants. A team of scientists led by Wang [4] identified genetic sequences expression of which has an effect on the amount of α-T in rice (*Oryza* spp.) and maize (*Zea mays* L.) cells. During the genome-wide search for associations, 1440000 high-quality single-nucleotide polymorphisms were used that encompass all plant chromosomes, and 13 genes were found, among which rice *VTE4* (*Osy*TMT) was identified as the main factor responsible for the content of α-T in all analyzed rice samples. Variation of the nucleotide sequence encoding *Osy*TMT, as well as the nucleotide polymorphism in the *Osy*TMT promoter region, had a pronounced effect on the change in the content of α-T. A similar result was obtained on soybean and maize plants after analyzing the effect of variations in the nucleotide sequence of *VTE4* in these plants on the content of α-T and the total amount of vitamin E [4, 25, 26]. Another study on maize [25], in combination of linkage mapping and GWAS, identified 32 significant loci controlling tocopherol content, 18 of which colocalized with the quantitative trait loci (QTLs). Authors found that tocopherol accumulation is affected by variations in non-tocopherol pathway genes, including genes involved in chloroplast function, metabolism of chlorophylls and fatty acids [25]. Similar analyses to identify the loci that affect the content of tocopherol were conducted on tomato, soybean and sweet corn [9, 27, 28]. It was identify that genes involved in starch synthesis could also affect tocopherol level [27].

Despite all the advantages of genetic transformation, consumers remain suspicious of the transgenic plants and prefer products from non-transformed organisms.

Accumulation of tocopherol under stressful conditions. Under natural conditions, plants are always exposed to a series of abiotic and biotic stresses, such as drought, salt, adverse temperatures, heavy metals, and various pathogens. Abiotic and biotic stresses induced rapid accumulation of reactive oxygen species (ROS) in plant cellular compartments. Imbalance ROS

in organelles results in oxidative stress in organisms and generates ROS signal that triggers the tocopherol biosynthesis which further affects the expressions of genes that regulate phytohormone biosynthesis and stress tolerance. The increased tocopherol enhanced the synergistic antioxidant effect of ascorbate-glutathione-tocopherol triad to eliminate the harm from stresses [29—31]. The increase in tocopherol can also stimulate the accumulation of osmotic substances under the adverse stresses. Taken together, these tocopherol effects combined to ultimately enhance plant stress tolerance [18, 21].

According to Collakova and DellaPenna [15], abiotic stress resulted in an 18- and 8-fold increase in total tocopherol content in wild-type and transgenic *Arabidopsis* plants, respectively. Non-stressed wild-type *Arabidopsis* leaves accumulated more than 95 % α-T and less than 5 % γ-tocopherol [15]. In stressful conditions, the alteration of α-T level takes places in two phases. In the first, its synthesis increases to eliminate ROS, preventing lipid peroxidation and maintaining stable redox status ensuring better plant cell protection against harmful oxidative effects. In the second phase, the content of net tocopherol decreases due to higher degradation that exceeds its synthesis under severe stress. The antioxidant response also depends on plant stress-tolerance, and frequently varies for different species. A clear manifestation of the first phase is an important feature of stresstolerant plants, whereas the second phase is typically evident in stress-sensitive ones [32].

Depending on the stress intensity and its duration, the content of synthesized antioxidants varies. Under severe, short light/temperature stress, the content of low-molecular weight antioxidants tend to decrease, which is correlated with an extra need for ROS scavenging. Under longer exposure of plants to unfavourable light and temperature conditions, the content of antioxidants gradually increase as a result of acclimation during long-term responses [33]. Therefore studying a wide range of plant species is a fruitful approach to investigating the natural variation in the antioxidant response of plants in relation to excess light and temperature.

Thus, the photosynthetic accumulation of low molecular weight antioxidants, including tocopherol, is crucially dependent on environmental factors such as light exposure, temperature, and water and nutrients supply. Changing environmental factors to extreme levels stimulates antioxidant defense and in some cases can contribute to the accumulation of tocopherol. The patterns of variation in the content of tocopherol depending on the conditions should be taken into account and used in the development of biotechnological regulations for the production of natural tocopherol.

High and low temperature effects on tocopherol in plants. Among the series stresses, high and low temperature turn to be critical factors on limiting plants productivity worldwide. Chilling often affects cell membrane fluidity by altering the composition of membrane fatty acids, thus influences the physiological and biochemical processes in plants [34]. On the other hand, high-temperature stress would induce the damage of cell membranes and accelerate plant senescence [30, 35]. High-temperature stress leads to reduced photosynthesis, nutrient deficiency [36], pigment degradation, and slow plant growth.

However, in several studies a positive association has been drawn between high temperature stress and enhanced synthesis/accumulation of tocopherols [37, 38]. Spicher et al. [39] suggested that massively increased concentrations of α-T in tomato plants subjected to rising temperatures is important for protection against high temperature stress and proper function of the photosynthetic apparatus.

Carrera and Seguin [37] observed higher α-T concentrations and lower of δ- and total tocopherol in warm environments (22.5 to 25.0 °C) compared to cooler ones (17.5 to 22.4 °C) [37]. These authors reported across 76 contrasted environments that α- and δ-tocopherol were linearly related to temperature, α-T increasing by 17.5 μg/g and δ-tocopherol decreasing by 35.2 μg/g oil per degree increase in temperature during the seed filling period. The simultaneous increase of α-T and decrease of δ-tocopherol in warm environments might be due to the temperature effect on the key enzyme γ-tocopherol methyl transferase (which methylates both γ- into α-, and δ- into β-tocopherol) or the gene encoding it [40]. Exposure of *Helianthus annuus* plants to a temperature of 35 °C induced high tocopherol levels at the reproductive stage and increased seed oil quantity [41]. In a study with lettuce, Tang et al. [42] observed that high temperature caused activation of tocopherol cyclase resulting in enhanced biosynthesis of vitamin E. Kumar, Singh and Nayyar [43] found that tocopherol content in wheat seedlings increased by 54 % at 30 °C but decreased by 69 % at 35 °C over content at the previous temperature.

At low temperature stress, tocopherol protects photosynthetic membranes from photooxidative stress [44, 45]. This finding supports the role of tocopherol at the gene level [42]. There is enough evidence that tocopherol content correlates positively with tolerance to low temperature in different plants [46, 47]. Chilling stress increased significantly α-T concentration in stressed *Medicago sativa* leaves. On average, concentrations range from 5 to 100 μg/g dry weight (DW). Total tocopherols concentrations found in the leaves at the start of chilling treatment are approximately 8.3±0.6 μg/g DW and increased significantly to 88.6±3.8 μg/g DW by the end of chilling treatment. After one day of the recovery period total tocopherol concentrations dropped to 48.4±5.8 μg/g DW, and by the end of experimental period declined to 27.6±0.6 μg/g DW, which was still higher than the control values [48].

A crucial role of tocopherols in plant low temperature acclimation is considered in several publications [33, 44, 49, 50]. An increase in α-T content has been reported in maize exposed to a long-term low temperature [33]. Tocopherols were promoted under low temperature stress. Results of Xiang et al. [51] showed that vitamin E gradually accumulated in response to low temperature stress (10 °C) but was limited by high temperature stress (40 °C) for sweet corn seedlings. The study speculated that the increased tocopherols in sweet corn seedlings were combined with polyunsaturated fatty acids to protect the integrity of plasma membrane under low temperature stress. At the same time, low temperature was supposed to slow down the metabolic reactions, together with darkness, influenced the accumulation of tocopherols and resulted in the gradual increase tendency of vitamin E.

Thus, because of tocopherols are very important in plant adaptation to low and high temperature stresses, their accumulation could modulate by variation of external factors [29]. Low temperature in combination with high light stress resulted in a fourfold increase in total tocopherol concentration in *Arabidopsis* [50].

Light-dependent tocopherol accumulation. Among other abiotic factors, a high light intensity stimulates the accumulation of antioxidant compounds in a various plant species [52]. The biosynthesis of tocopherols in photosynthetic plant cells is also light-dependent [53], and increased during senescence [54]. As reported Zhang et al. [55] total tocopherol content demonstrated a net increase in seedlings *Brassica napus* L. growing under illumination while no changes were detected in dark. Under high light conditions the increase in α-T concentration was found in *C. reinhardtii* [53], while in tobacco leaves high light induces even 2 times higher tocopherol levels [56]. However, in some cases, high light diminishes the α-T content [29]. In maize leaves exposed for up to 24 h to high light (1000 μmol/(m^2 · s) at low temperature (5 °C) a 50 % decrease of α-T was observed [57]. On the other hand, the content of tocopherol increased by 18 times when plants were subjected to a combination of nutrient deficiency and high-light stress (800—1000 μmol/(m^2 · s)) [15].

Changes in the content of tocochromanols are related to light stress intensity [58—60]. It is known that the α-T concentration tends to decrease when stress is severe and the amount of ROS in chloroplasts increases [32].

An analysis of *Arabidopsis* plants grown in both low and high light revealed that high light conditions dramatically increased the content of α-tocopherol [60]. The most abundant tocopherol in *Arabidopsis* was α-T with levels in non-induced plants that varied between 10 and 15 μg/g fresh weight (FW). The corresponding levels of γ-tocopherol, the biosynthetic precursor, were 0.15—0.3 μg/g, reaching only between 1.5 and 3 % of those of α-T ocopherol. [61]. An increasing accumulation of α-T as a sign of light adaptation was observed in *Arabidopsis*. The level of α-tocopherol increased > 4-fold. In young rosette leaves, the increase in the α-tocopherol level was considerably lower. In the vte4 mutant, γ-tocopherol substitutes for α-tocopherol, and a similar response of γ-tocopherol to that of α-tocopherol in the WT under high light, in both old and young leaves, can be observed [54].

When untreated plants were kept under continuous illumination with white light, a slow rise in α-T levels could be seen, so that after 48 h levels had increased by 30—40 %. Levels of α-T were much lower in the wild-type plant; however, in stress situations, caused by wounding or chemicals, level of α-T changed very quickly. Exposing plants to UV light for 6 h had only minor effects on α-T levels, but led to considerable enhancement of α-T concentrations up to 1.5 μg/g FW under subsequent continuous illumination. The increase was only moderate as long as plants were exposed to UV light, but accelerated after transfer to normal light [61].

Tocopherol accumulation under water deficit. The role of tocopherol in plant adaptation to water deficit have been studied in several plant species, and they reveal the positive relationship between tocopherol biosynthesis/accumulation and water stress [62—64].

Unstressed field-grown rosemary (*Rosmarinus officinalis*) showed α-T concentration ranging from 2 to 35 μg/g DW depending on plant water status and its 15-fold increase in drought-subjected plants [63]. α-Tocopherol levels increased also in sage balm (*Salvia officinalis*) and lemon balm (*Melissa officinalis*) leaves under drought stress [59]. A high amount of α-tocopherol is intrinsically produced in *Arabidopsis* and tobacco [62]. Liu et al. [62] observed that transgenic tobacco plants overexpressing the *VTE1* gene enhanced α-T synthesis resulting in better protection to water deficiency. Total tocopherol levels increased in a near linear manner with the progress of drought treatment in both wild type and transgenic plants [62]. Overexpression of *VTE1* resulted in substantial accumulation of other tocopherol isoforms than α-T, and significantly increased drought tolerance of transgenic plants by almost 50 % in terms of wilted plant rate and relative water content of leaves [21, 62]. The proportion of α-T in transgenic plants overexpressing *VTE4* was dramatically increased by up to 97.1 %, accompanied by only a slight increase in total tocopherol content [20].

Another strategy of genetic manipulation tocopherol content is to identify suitable promoters to avoid negative effects at drought stress. For example, it was observed that transgenic tomato plants expressing *VTE2* (*Solanum chilense*) under a stress-inducible promoter exhibited increasing level of α-T with growing time under drought stress [64]. Increased tocopherol content was evident in both WT and transgenic plants after 9 days of drought treatment. While WT plants exhibited a two-fold increase in the content of α-T, the transgenic lines showed an additional increase (up to around 5-fold) at the last day of measurement (20th day). Although α-T content also exhibited a continuous increment in all plants under drought, the levels were not significantly different among most genotypes. It should be noted that the absolute levels of α-T were approximately 10-fold higher than those of γ-tocopherol.

Tocopherol accumulation in plant cells *in vitro*. Various strategies using plant *in vitro* systems have been successfully exploited to improve the production of valuable plant substances. Apart from transgenic research, conventional plant cell cultures have been successfully used for the production of several specific metabolites. Production of these compounds through plant cell culture system will ensure a continuous supply of uniform quality, highly specialized and natural components that cannot be produced in equal quality and specificity by other means [65]. Compared to agricultural production, cell suspension cultures are independent on seasonal and environmental factors, and overcomes the challenges of biosafety [66, 67].

Several studies have shown that tocopherols can be accumulated in callus plant tissue cultures obtained from *Arabidopsis thaliana* [68], *Carthamus tinctorius* [69—71], *Elaeagnus angustifolia* [72], *Nicotiana tabaccum* [18], *Amaranthus caudatus,* and *Chenopodium quinoa* [73].

The most investigated system for tocopherol production *in vitro* is sunflower (*Helianthus annuus* L.) cell cultures [68, 69, 74—76]. Heterotrophic sunflower cell cultures have been shown to be a suitable *in vitro* natural α-T production system of [69, 71]. Increasing metabolite production by plant cell cultures was achieved by special supplements, including precursor

feeding and elicitor application. Supplementing the cells with precursors enhances the availability of respective substrates and overexpressions of rate-limiting genes are also very helpful for the same reason.

As Furuya et al. [71] reported, the addition of the precursor of the hydrophobic tail, phytol, increased the total tocopherol content in *C. tinctorius* callus by some 18-fold. Although the callus produced α-T as the major component and β-tocopherol as the minor, the control of phytol led to the accumulation of α-, β-, γ-, and δ-tocopherol [71]. Biosynthetic precursor of the polar head, homogentisic acid (HGA), was only slightly effective in sanflower cells [71].

By contrast, when 100 mg/l homogentisic acid was added to sunflower suspension cultures, a clear stimulatory effect (30 % increase) on the production of α-T was observed, suggesting that this substance can be useful for improving the yield of sunflower cell cultures. The administration of higher homogentisic acid concentrations (up to 200 mg/l) did not induce a further increase in α-T levels. Phytol alone did not increase α-T production. When added with HGA, it did not significantly alter the HGA-dependent stimulation of α-T production [69].

These findings imply that modulation the vitamin E metabolic pathway could enhance α-T levels in cell suspensions. Tocopherol biosynthetic precursors (tyrosine, 4-hydroxypyruvic acid, HGA and phytol) that are involved in the upstream biosynthesis of vitamin E pathway were supplemented in suspension cultures of tobacco and studied in detail [18]. The combination of HGA 150 μM + phytol 150 μM showed the highest (36-fold) response, whereas individual precursors did not show any drastic increase in α-tocopherol. These effective responses could be due to the precursors of the shikimate pathway, which when combining with phytol (derived from chloroplasts, which is abundant in green tissue) helps in channeling the metabolic flux more effectively. As phytol is known to provide the phytyl tail moiety to aromatic groups, which by itself would be a limiting factor in tocopherol biosynthesis [18].

In higher plants, the biosynthesis of α-T is localized in plastids and more specifically in chloroplasts of photosynthetic tissues [59]. Due to this capacity, photoautotrophic cell cultures are also relevant for the biotechnological application in combination with mass cultivation [76, 77]. Only limited number of plant species are capable of accumulating biomass in photoautotrophic cultivation *in vitro* in the absence of any reduced carbon source. Photomixotrophic cultures, that are photosynthetically active, are available from some species but require the presence of exogenous sugars in the culture medium [76—78].

Caretto et al. [69] noticed that non-green sunflower callus cultures seemed to have constantly lower levels of tocopherols than green callus cultures. Fachechi et al. [74] showed that in cells with a higher content of α-tocopherol, the level of chlorophyll was higher. The authors showed also a positive correlation between the photosynthetic potential of sunflower cell cultures and their α-T biosynthetic capability. Photomixotrophic cultures are photosynthetically active but require the presence of exogenous sugars in cultural medium. They combine the advantages of plant suspension cultures with carbon autotrophy. However, the *in vitro* photosynthetic potential of plant cells is affected by the concentration of sugar in the cul-

tural medium, since sucrose is known to reduce the level of chlorophyll and, therefore, photosynthesis [77]. Therefore, the cultivation regime should be optimized by adjusting hormonal conditions and reducing the amount of sugar to obtain photomyxotrophic growth. Consequently, preliminary experiments are necessary to determine the metabolic and hormonal conditions that contribute to the highest yield of phytometabolites. Fachechi et al. [74] used a ten-fold reduction in sucrose in the culture medium of cell suspensions, and obtained a photomyxotrophic cell line. The authors showed that these cells had an increased content of chlorophyll and a number of chloroplasts. In a similar way, photomixotrophic growth of sunflower suspension cultures was obtained modulating light intensity, sugar concentration and age of culture. Under these conditions, the accumulation of α-T increased up to 230 % that of heterotrophic cell cultures [76].

Plant cell cultures are always characterized by a certain level of variability, within which it is important to identify highly productive cell lines [74]. Cell line selection is an important step in developing a cell culture as a «cell factory» to produce a valuable compound.

To stimulate the tocopherol production by plant cells *in vitro,* abiotic and biotic elicitors have been successfully used. A considerable enhancement of α-T production was achieved, both in sunflower and *Arabidopsis thaliana* cell cultures, by the exogenous application of 5 μM jasmonic acid [68] or by hypoxic conditions [69]. Jasmonic acid (JA) is the quickest and critical signal molecule of complex signaling networks in plants which plays important role in protection against biotic and abiotic stresses. The exogenous application of JA is known to elicit the production of a wide range of compounds by inducing the expression of plant genes for various biosynthetic pathways, through enhanced production of defense-related molecules, including tocopherol [61]. Gala, Mita & Caretto [68] reported that the addition of JA to the culture medium enhanced the α-T accumulation. The highest increase of tocopherol (49 %) was found in cultures treated with 5 mM JA. Probably, the observed enhancement of tocopherol production in sunflower and *Arabidopsis* cell cultures could be due to the ability of the exogenously added JA to upregulate the biosynthetic pathway through the induction of gene expression. Sandorf and Hollander-Czytko [61] showed that the treatment of *A. thaliana* plants with methyl jasmonate (MJ) led to the induction of a tyrosine aminotransferase (TAT) – the first enzyme in the biosynthetic pathway leading via homogentisic acid to plastoquinone and tocopherols. Gene expression analysis revealed that the levels of two genes of the tocopherol biosynthetic pathway, *p*-hydroxyphenyl pyruvate dioxygenase and homogentisate phytyltransferase, were indeed enhanced, compared with the control actin gene, after the jasmonic acid treatment of *Arabidopsis* cell cultures [75].

Badrhadad et al. [72] examined the effects of JA and salicylic acid (SA) elicitors on production of α-T in cell suspension cultures of *Elaeagnus angustifolia* that are naturally rich in tocopherols. Authors observed that tocopherol content in *E. angustifolia* callus was increased by about 2.5 times after addition of 25 μM JA as compared to control. The highest increase of tocopherol was found in this cell culture treated by 1 mM SA.

Antognoni et al. [73] studied calli from *Amaranthus caudatus* and *Chenopodium quinoa*, two pseudocereals that naturally produce tocopherols, and analysed regulation role of the elicitors coronatine and MJ in tocopherol biosynthesis. In *A. caudatus* cultures, treatment with 100 μM MJ increased the production of α-T up to 5-fold, thus reaching levels close to those found in seeds. [73]. This increase in α-T was associated with a proportional increase in TAT activity. By contrast, in *C. quinoa* cultures, elicitation with MJ did not have any effect, neither on tocopherol production, nor on TAT activity. The differential response in the two species indicates that TAT is a crucial checkpoint, thus confirming previous data from *Arabidopsis* [61].

Besides abiotic effectors, the administration of biotic elicitors could activate the production of tocopherols. Chavan et al. [70] examined the effects of the fungal strains *Trametes versicolor*, *Mucor* sp., *Penicillium notatum*, *Rhizopus stolonifer*, and *Fusarium oxysporum* on cell growth and α-T productions in *Carthamus tinctorius* cell cultures. Addition of *T. versicolor* (50 mg/l) significantly improved α-T production (12.7-fold), whereas other fungal strains were not effective.

Srinivasan et al. [79] developed a high α-T yielding cell line of *H. annuus,* using a model based on metabolic engineering approach. Having identified and ranking suitable enzyme targets for overexpression, authors adapted an available genome-scale model of *Arabidopsis* for simulating *H. annuus* using constraint-based analysis. The enzyme HPPD was chosen for experimental validation as it was the top-ranked enzyme target predicted by FSEOF (Flux Scanning-based Enforced Objective Flux). Experimental validation of the top strategy (overexpression of HPPD) resulted in a high α-T yielding transformed cell line (up to 240 μg/g), which was ≈10-fold more than in the untransformed cell line. A cell suspension was developed from the selected transformed cell line for *in vitro* production of α-tocopherol, which resulted in a maximum yield of 412.2 μg/g and titre of 6.4 mg/l . The α-T yield obtained in this study is even higher than the previously reported highest α-T yield (80 μg/g) in a photomixotrophic cell suspension of *H. annuus* [76]. This demonstrates the potential of model-based (rational) metabolic engineering to maximize α-T yield in *H. annuus* plant cells which can further be adapted to other plant species.

As plant cell *in vitro* technologies developed, new effective approach are becoming available including application of genetic modifications. So, Harish et al. [18] applied two approaches for increasing the α-T content in tobacco: (1) transgenic approach, by constitutive overexpression of the genes encoding *Arabidopsis* homogentisate phytyltransferase (HPT) and tocopherol cyclase (TC, *VTE4*) through *Agrobacterium*-mediated genetic transformation; (2) non-transgenic approach, by supplementation of intermediates/precursors of vitamin E biosynthesis like tyrosine, *p*-hydroxyphenyl pyruvic acid, homogentisic acid (HGA) and phytol in different concentrations and combinations using cell suspension culture system. The α-T content in transgenic plants expressing HPT and TC increase by 5.5 and 4.1-fold, respectively, over the wild type [18]. In the second approach, the supplementation of precursors in cell suspension cultures, i.e., combination of 150 μM HGA + 100 μM phytol, showed the maximum enhance-

ment of α-tocopherol (36-fold). These findings clearly imply that enhancement of α-T levels in tobacco system is possible, if we could modulate the vitamin E metabolic pathway. This is a very useful finding for the large-scale production of natural vitamin E. Among the two systems tested, cell suspension culture-based system is ideal over the transgenic technology due to its efficiency and no biosafety concerns. The effectiveness of precursor feeding to tobacco cell suspension culture to produce more α-T in non-transgenic mode indicates the promise of this simple plant cell culture-based methodology for the large-scale production of natural nutritional and therapeutic component without any biosafety concerns.

Thus, application of the combination of biotechnology techniques, genetic engineering and optimization of cultivation conditions, greatly stimulates the accumulation of α-T in photosynthetic organisms.

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Received 25.11.2021

СТРАТЕГІЇ ПІДВИЩЕННЯ ВМІСТУ АЛЬФА-ТОКОФЕРОЛУ В РОСЛИНАХ

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Основними способами отримання α-токоферолу (α-Т) є хімічний синтез та екстракція α-Т з рослинних олій. Широко використовувана синтетична форма під назвою all-rac-α-токоферол складається із суміші восьми стереоізомерів, при цьому частина природного стереоізомеру RRR-α-токоферолу становить усього 12,5 %. Природний α-Т в 1,5 раза активніший за синтетичні форми, тому пошук ефективних джерел природного α-Т триває. Рослинні олії з насіння соняшника, кукурудзи, ріпаку та сої є основними джерелами натурального комерційного вітаміну Е з низькою активністю через низький вміст α-Т. В багатьох дослідженнях показано зростання накопичення α-Т у клітинах рослин за зміни умов культивування: інтенсивності світла, фотоперіоду, рівня азоту, температури, типу вуглецевого живлення тощо. Стресові умови стимулюють накопичення антиоксидантів у фотосинтезуючих організмах, але можуть обмежувати нормальну швидкість їх росту. Генна інженерія дає змогу створювати рослини з високим вмістом α-Т введенням кодувальних послідовностей (CDS) значущих генів шляху синтезу токохроманолу в ядерний геном трансгенних рослин. CDS кДНК ключових ферментів синтезу α-Т, таких як гомогентизатгеранілгеранілтрансфераза (HGGT), токоферолциклаза (TC), γ-токоферолметилтрансфераза (γ-MTM) з рису, сої, кукурудзи, моркви тощо, використовують для збільшення загального вмісту токохроманолів. Комбінуванням біотехнологічних методів, генної інженерії та добором умов культивування можна значно стимулювати накопичення α-Т у фотосинтезуючих організмах.

Ключові слова: α-токоферол, вітамін Е, токохроманол, біотехнологія, трансгенні рослини.