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Engineering tocopherol biosynthetic pathway in *Arabidopsis* leaves and its effect on antioxidant metabolism

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ABSTRACT

With genetic manipulation, five genes (*HPPD*, *VTE2*, *VTE3*, *VTE1*, and *VTE4*), which encode enzymes involved in tocopherol biosynthesis, were over-expressed in model plant *Arabidopsis thaliana*, either alone or in couple combinations (*VTE2* + *VTE4* and *VTE3* + *VTE4*), to value and compare the roles of enzymes played in tocopherol biosynthetic pathway under the same genetic background. Our results suggested that, elevated expression level of biosynthetic pathway gene affected either total tocopherol content or composition, it is recommended to choose two or more enzymes with different functions for genetic manipulation. It was also found that metabolic engineering of tocopherol biosynthetic pathway affected endogenous ascorbate and glutathione pools in leaves. Further study suggested that expression levels of genes encoding enzymes of Halliwell–Asada cycle were up-regulated, such as *APX*, *DHAR* and *MDAR*. These findings provide hints on the relationship of lipid-soluble antioxidant vitamin E and water-soluble antioxidants vitamin C and glutathione, which will help to perfect theory in plant physiology and give practical instruction for metabolic engineering.

1. Introduction

Vitamin E is an essential nutrient of daily diet for humans and animals. Naturally occurring vitamin E exists in eight chemical forms (α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol) that have varying levels of biological activity. The nutritional values of vitamin E were affirmed in 1922 [1]. The National Institutes of Health (NIH) currently suggests a recommended daily allowance (RDA) of 15–19 mg α-tocopherol for adults (http://ods.od.nih.gov/factsheets/vitamine.asp). Higher daily vitamin E doses have been needed for cancer reduction, immune response, and cardiovascular benefits [2]. Among the family, α-tocopherol is believed to have the highest vitamin E activity to meet human requirements, and it is preferentially retained and distributed throughout the body [3]. Naturally synthesized α-tocopherol, which is a single (*R*, *R*, *R*) stereoisomer, has more activity than chemically synthesized α-tocopherol [4].

Tocopherols can be synthesized only in photoautotrophy organisms, including plants and other oxygenic, photosynthetic organisms. Although the tocopherol biosynthetic pathway had been elucidated from 1979 [5], the genetic analysis of the pathway and key enzymes had only commenced since 1990s, with the approaches of genetic and genomics-based methodologies in the model organisms *Arabidopsis thaliana* and *Synechocystis* sp. PCC6803.

Tocopherol biosynthesis mainly takes place in plastids of higher plants. The tocopherol biosynthetic pathway utilizes two compounds from different metabolic pathways as precursors, which include homogentisic acid (HGA), derived from cytosolic shikimate metabolic pathway for head group and phytyldiphosphate (PDP) [6] from the plastidic methylerythritol phosphate (MEP) pathway for tail synthesis [7,8] (Fig. 1). There are at least five enzymes involved in the biosynthesis of tocopherols, excluding the bypass pathway of phytyl-tail synthesis and utilization (Table 1). HGA is

Abbreviations: APX, ascorbic acid peroxidase; AsA, ascorbic acid (vitamin C); DHA, dehydroascorbic acid; DHAR, dehydroascorbic acid reductase; GDPME, GDP-D-mannose-3, 5-epimerase; GDPMPPase, GDP-D-mannose pyrophosphorylase; GSH, glutathione; GSSG, oxidized glutathione; HGA, homogentisic acid; HPLC, high performance liquid chromatography; HPPD, *p*-hydroxyphenylpyruvic acid dioxygenase; L-GalDH, L-galactose dehydrogenase; L-GalPPase, L-galactose 1-P phosphatase; L-GLDH, L-galactono-γ-lactone dehydrogenase; MDAR, monodehydroascorbic acid; MEP, methylerythritol phosphate; NC, non-transgenic control; PDP, phytyldiphosphate; PQ8, plastochromanol-8; PUFAs, protect polyunsaturated fatty acid chains; RDA, recommended daily allowance; ROS, reactive oxygen species; VTC2, GDP-L-galactose phosphorylase; VTE1, tocopherol cyclase; VTE2, homogentisate phytyltransferase; VTE3, 2-methyl-6-phytylplastoquinol methyltransferases; VTE4, γ-tocopherol methyltransferase.

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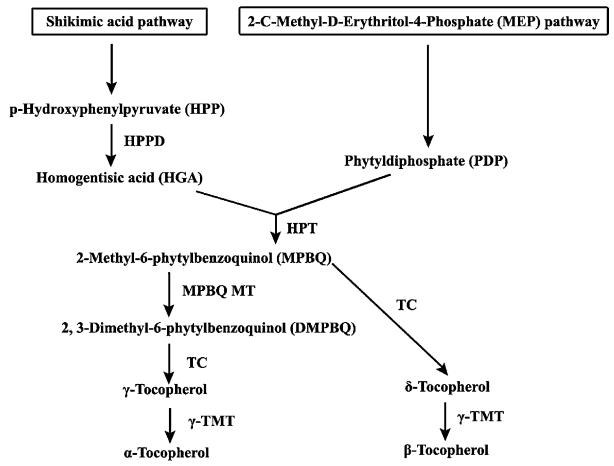

Fig. 1. Simplified tocopherol biosynthetic pathway from shikimate and MEP pathways.

produced from the tyrosine aromatic amino acid catabolite *p*-hydroxyphenylpyruvate (HPP) by the cytosolic enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD) [9]. Condensation of HGA and PDP is catalyzed by homogentisate phytyltransferases (VTE2) [10]. The product of this reaction, 2-methyl-6-phytylbenzoquinol (MPBQ), is the first phytylquinol intermediate in the pathway and can be methylated to 2,3-dimethyl-6-phytyl-1, 4-benzoquinol (DMPBQ) by MPBQ methyltransferase (VTE3) [11]. Both MPBQ and DMPBQ are substrates for tocopherol cyclase (VTE1) to yield the first tocopherols of the pathway, δ-tocopherol and γ-tocopherol, respectively [12]. Both δ- and γ-tocopherol can be methylated by γ-tocopherol methyltransferase (VTE4) to yield β- and α-tocopherol, respectively [13].

As a member of plant secondary metabolites, vitamin E has various biological and pharmaceutical functions to humans as well as to plants. Crops and vegetables are the best source for natural vitamin E. Nevertheless, vitamin E is of low content, and the composition of the eight forms needs to optimize. Recently, metabolic engineering has been widely applied in order to achieve higher yields of specific metabolites. The in-depth understanding of biosynthetic pathways, along with the increasing number of cloned genes involved in biosynthesis, enable the exploration of metabolic engineering as a potential effective approach to increase the yield of specific metabolites by enhancing rate-limiting steps or by blocking competitive pathways [14]. Increasing vitamin E content and α-tocopherol composition in vegetables and crops has been an important aim for metabolic engineering. Strategies as to over-expressing different genes involved in the pathway have been employed. Although significant work has been done [15], it is still hard to assess the relative importance of each enzyme in tocopherol biosynthetic pathway, due to different genetic background and various manipulation used in former studies. Valuation of the contribution of different enzymes under the same genetic background will be essential to provide effective strategies for large-scale commercial production of biosynthetic tocopherol. In this work, genes encoding these enzymes were cloned from the model plant *A. thaliana* and constitutively over-expressed, alone or in combination, in order to achieve this assessment.

In metabolic engineering, when target nutritional product is increasing, other related nutritional products in the same bioreactor might be affected, in some cases they would decrease. Many studies reported the antioxidant properties of tocopherols, such as photo protection and reduction of lipid peroxidation by reducing lipid peroxyl radicals to their corresponding hydroperoxides [16,17,18]. As lipid-soluble antioxidant, tocopherols locate mainly on membranes of many cellular compartments, and will be oxidized to tocopherol radical [19]. There are two important water-soluble antioxidants in plant cells—ascorbic acid (vitamin C, AsA) and glutathione (GSH), and they can scavenge reactive oxygen species by Halliwell–Asada cycle during normal metabolism and particularly during stress [20]. Especially vitamin C is not only an important antioxidant in plant physiology, but also a metabolic product with important nutritional and physiological values for humans and animals. Former works indicated there might be relationship existing among tocopherol, AsA and glutathione contents [21,22]. It was reported [22] that deficiency in one antioxidant in tocopherol, AsA or glutathione led to increased oxidative stress and the concomitant increase in alternative antioxidants. In some other cases, such as sunflower cell lines, high content of tocopherols leads to higher content levels of ascorbate and glutathione pools [21]. However, in the study which observed the plant responses to oxidant stress in the presence of Cu or Cd [23], the correlation between high tocopherol and low ascorbate/glutathione levels was not seen.

In this study AsA and glutathione contents in transgenic lines over-expressing tocopherol biosynthetic pathway genes were analyzed in order to assess whether accumulation of vitamin E affected other antioxidants in plant. Furthermore, some studies were done in order to explain the change of vitamin C and glutathione in transgenic lines.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of wild-type *Arabidopsis* (Columbia ecotype) were sterilized with chlorox and spread on Murashige and Skoog (1962) plates, then treated at 4 °C for 3–5 days and induced for germination in 24 h-white-light for 6 days. Then the seedlings were transferred into soil at 20 °C under 16-h photoperiod of light at 120 μmol m^{-2} s^{-1}.

2.2. cDNA generation and vector construction

For construction of plant expression vectors, *myc* tag was used as screening labels and sub-cloned into *Xho*I and *Pst*I sites of pBluescript SK+ vector (pBS; Stratagene) to form the vector pBSmyc (a gift from Prof. Hongquan Yang, SIPPE, CAS). Total RNA was isolated from leaves of *Arabidopsis thaliana* (Columbia ecotype) by using TRIzol reagent (GIBCO/BRL). The cDNAs of

Table 1
Enzymes, basic functions, loci and genes encoding tocopherol biosynthetic enzymes in *Arabidopsis thaliana*.

Pathway enzyme	Basic function	*Arabidopsis*	
		Gene	Locus
p-Hydroxyphenylpyruvic acid dioxygenase (HPPD)	Head group synthesis	*PDS1*	At1g06570
Homogentisate prenyltransferase (HPT)	Prenylation of HGA	*VTE2*	At2g18950
2-Methyl-6-phytylbenzoquinone methyltransferase (MPBQ MT)	Methylation of MPBQ and MGGBQ	*VTE3*	At3g63410
Tocopherol cyclase (TC)	Cyclization	*VTE1*	At4g32770
γ-Tocopherol methyltransferase (γ-TMT)	Methylation of δ- and γ-tocopherol	*VTE4*	At1g64970