Genetic modification of tocopherol biosynthesis in oilseed rape (*Brassica napus* L.) for nutritional purposes

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ABSTRACT

Genetic engineering offers the possibility to modify plant storage lipids and valuable secondary compounds in order to meet specific nutritional and even therapeutic requirements. The current trend in genetic modification of oil and fatty acids of oilseed crops, especially oilseed rape (*Brassica napus* L.), further increases the importance of tocopherols (TOC) and tocotrienols, which are the most powerful lipid-soluble antioxidants. The former occur in rapeseed oil as a mixture of two predominant forms, α-TOC and γ-TOC, which differ in their bioactivity (vitamin E) and antioxidant properties. In the course of a metabolic engineering approach, the strategy is to elevate tocopherol levels by increasing the flux through the pathway by heterologous over-expression of enzymes that catalyse relevant steps in TOC biosynthesis, such as 4-hydroxy-phenylpyruvate dioxygenase (HPPD) and homogentisate phytyltransferase (HPT).

Key words: Tocopherol - nutrition - oilseed rape - *Brassica napus* – transgenic approach

INTRODUCTION

With the advent of so-called "functional food", the benefits of scientific research are now reaching the consumer. Genetic engineering offers the possibility to modify plant storage lipids and valuable secondary compounds in order to meet specific nutritional and even therapeutic requirements. The goal of the German collaborative project 'NAPUS 2000' is the multi-faceted improvement of rapeseed (*B. napus*) quality for human nutrition, including the augmentation of the content of tocopherols (cf. Leckband *et al*. 2002). These lipoid antioxidants are present in oil seeds, leaves and other green parts of higher plants. Since vitamin E is only synthesised by most photosynthetic organisms (Fig. 1), it is a very important dietary nutrient for humans and animals (cf. Schultz 1990, Bramley *et al*. 2000). Aside of a total increase of TOC content, one of the goals is to genetically enhance the α-TOC content in rapeseed oil in order to improve natural vitamin E supply. At the same time it is important to achieve better oxidative stability of unsaturated fatty acids through an increase of γ-TOC content (Raclaru *et al*. 2002).

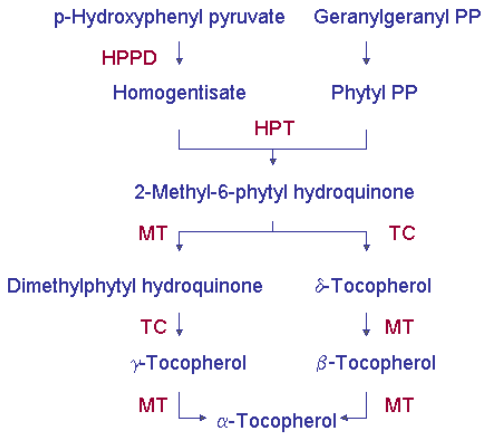

Fig. 1. Scheme of tocopherol biosynthesis in plants: HPPD=4-hydroxyphenylpyruvate dioxygenase, HPT=homogentisate phytyltransferase, MT=methyl transferase, TC= tocopherol cyclase (cf. Schultz 1990, Bramley 2000).

MATERIALS and METHODS

Hypocotyl segments of spring rapeseed cv. 'Drakkar' were transformed using a protocol as described earlier (Zarhloul *et al*. 1999). The *Agrobacterium tumefaciens* strain ATHV C58C1 (cf. Hellens *et al*. 2000) harbouring constructs with different combinations of seed-specific

promoters (DC3 derived from *Daucus carota* including an Ω-leader sequence, napin promoter) and genes of interest, *viz*. two dioxygenase genes of different origin (HPPD1, HPPD2) and an *Arabidopsis thaliana* phytyltransferase (HPT) as single construct and in combination with HPPD2 (tandem construct HHD), was used for the transformation procedure. The binary vector used was pPZP111 (Hajdukiewicz *et al*. 1994) with the neomycin phosphotransferase (NPTII) gene as selectable marker. Successful gene transfer was initially confirmed by NPTII ELISA assays (5 Prime-3 Prime Inc., Boulder, USA; Agdia Inc., Elkhart, Indiana, USA) of T1 plantlets. Only those T1 plants with extinction values ≥0,2 were maintained, transferred to soil and cultivated in three temporally and spatially different greenhouse environments (average temperature 17-20 °C, 16-17 hours day light). Following the extraction of T2 seeds (0.3 g pooled seeds) with petroleum ether the TOC composition was determined by HPLC and fluorescence detection as described by Thies (1997), with β-TOC as internal standard and iso-octane as sample solvent. Asides the main TOC species (α-TOC, γ-TOC) rapeseed oil contains minor amounts of δ-TOC und plastochromanol-8. Based on seed oil content (91% dry matter, NIRS method) total tocopherol content is expressed as mg/kg seed.

RESULTS and DISCUSSION

Genetic engineering is applied to modify relevant genes of the TOC biosynthetic pathway in order to create novel genetic variation for these traits. In a first step we investigated the effect of overexpressing heterologous HPPD and HPT genes in spring canola. Interest in HPPD has raised due to its function as target enzyme in the biosynthesis of both plastoquinones and tocopherols acting as essential elements of the photosynthetic electron transport chain and of the antioxidant system, respectively (cf. Tsegaye *et al*. 2002). HPT activity, responsible for the condensation of homogentisate and phytyl diphosphate (Fig. 1), is a limiting, committed step of tocopherol biosynthesis in plants (cf. Collakova and DellaPenna 2003). Etiolated hypocotyl segments of double-low spring rapeseed cultivar 'Drakkar' were genetically transformed using the *A. tumefaciens* high virulence strain ATHV C58 C1 harbouring different chimeric HPPD or HPT single gene constructs (Fig. 2).

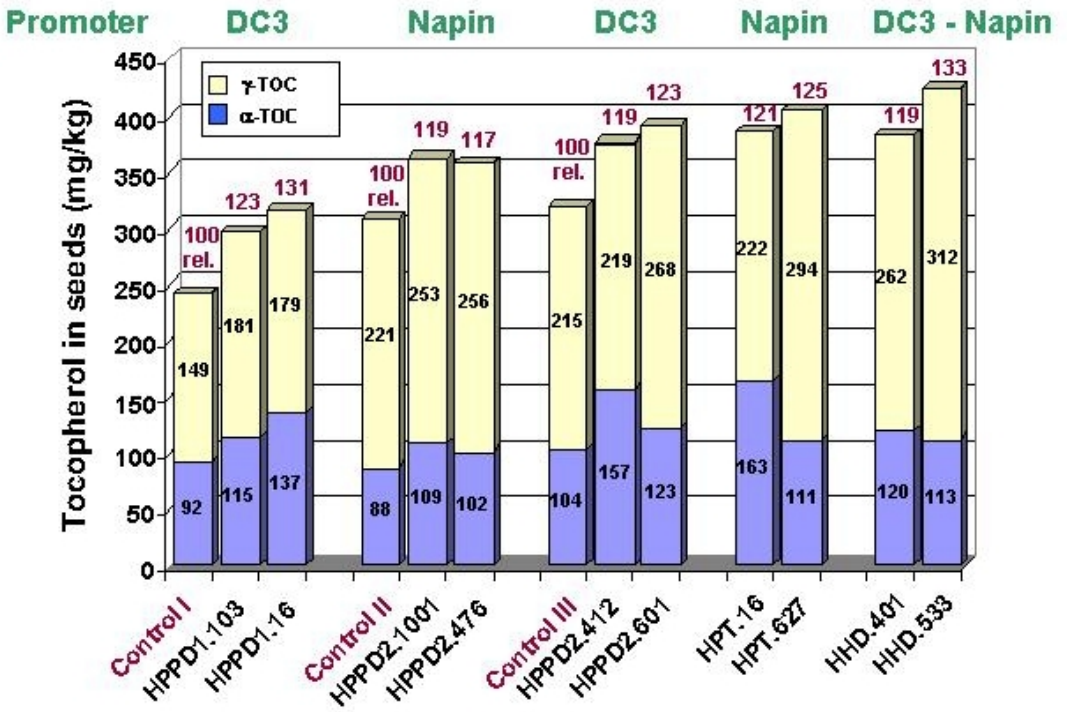

Fig. 2. Effects of heterologous overexpression of single TOC genes (HPPD1_DC3, HPPD2_DC3, HPPD2_Napin, HPT_Napin) or the tandem construct (HHD) on tocopherol composition of transgenic oilseed rape (cv. 'Drakkar') as compared to the average of the control plants cultured in three different environments.

In order to optimise TOC synthesis in canola seeds we conducted further modifications in the transformation experiments using different dioxygenase gene-promoter variants (HPPD1_DC3, HPPD2_DC3, HPPD2_Napin) and the HHD tandem construct comprising a combination of HPPD2_DC3 and HPT_Napin. Seed oil was extracted from pooled T2 seed samples and the composition of tocopherols was determined by HPLC. The differences in seed TOC level observed between control plants derived from the three environments are attributed to variations in plant growth conditions during seed development and oil synthesis. As compared to the average of the respective control plants the best HPPD transformants showed an increase of about 30% in total seed TOC content (Fig. 2). Surprisingly, the napin promoter in the HPPD2 gene constructs showed no higher increase in TOC content than the DC3 promoter. It seems that the outcome of HPPD overexpression is biochemically limited and tightly controlled *in vivo* due to degradation of homogentisate, which is considered as a highly reactive intermediate compound (Fernández-Cañón and Peñalva 1995, Hiraku *et al.* 1998). Regarding the modification of HPT by using either the single or a tandem construct in combination with HPPD our results are preliminary as the number of transformants was presently not sufficient. Similar results - a moderate enhancement (30-40%) of seed TOC content - have been reported for genetically modified *A. thaliana* attempting to increase the flux of the TOC biosynthetic pathway by altering the level of the same single rate-limiting enzyme (HPPD, HPT) activities (Tsegaye *et al*. 2002, Collakova and DellaPenna 2003). The fine regulation of TOC biosynthesis in plants is largely unknown as pool size and composition are subject to large changes in response to many factors including environmental effects and stresses (temperature, light, water and nutrient availability), the development of the plant and senescence (cf. Munné-Bosch and Alegre 2002).

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REFERENCES

Bramley, P.M., I. Elmadfa, A. Kafatos, F.J. Kelly, Y. Manios, H.E. Roxborough, W. Schuch, P.J.A. Sheehy, and K.-H. Wagner, 2000: Vitamin E (Review). J. Sci. Food Agric. 80, 913-938.

Collakova, E., and D. DellaPenna, 2003: Homogentisate phytyltransferase activity is limiting for tocopherol biosynthesis in *Arabidopsis*. Plant Physiol. 131(2), 632-642.

Fernández-Cañón, J.M., and M.A. Peñalva, 1995: Molecular characterization of a gene encoding a homogentisate dioxygenase from *Aspergillus nidulans* and identification of its human and plant homologues. J. Biol. Chem. 270, 21199-21205.

Hajdukiewicz, P., Z. Svab, and P. Maliga, 1994: The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant Mol. Biol. 25 (6), 989-994.

Hellens, R., P. Mullineaux, and H. Klee, 2000: A guide to *Agrobacterium* binary Ti vectors. Trends Plant Sci. 5 (10), 446-451.

Hiraku, Y., M. Yamasaki, and S. Kawanishi, 1998: Oxidative DNA damage induced by homogentisic acid, a tyrosine metabolite. FEBS Lett. 432, 13-16.

Leckband, G., M. Frauen, and W. Friedt, 2002: NAPUS 2000. Rapeseed (*Brassica napus*) breeding for improved human nutrition. Food Research International 35, 273-278.

Munné-Bosch, S., and L. Alegre, 2002: The function of tocopherols and tocotrienols in plants. Crit. Rev. Plant Sci. 21 (1), 31-57.

Raclaru, M., W. Lühs, M.K. Zarhloul, J. Gruber, R. Sadre, D. Weier, M. Frentzen und W. Friedt, 2002: Entwicklung von neuartigen Raps-Genotypen (*Brassica napus* L.) mit genetisch veränderter Tocopherol-Zusammensetzung. Vortr. Pflanzenzüchtg. 54, 341-344.

Schultz, G., 1990: Biosynthesis of α-tocopherol in chloroplasts of higher plants. Fat Sci. Technol. 92, 86-90.

Thies, W., 1997: Quantitative Bestimmung der Tocopherole durch HPLC. Angew. Bot. 71, 62-67.

Tsegaye, Y., D.K. Shintani, and D. DellaPenna, 2002: Overexpression of the enzyme *p*-hydroxyphenol-pyruvate dioxygenase in *Arabidopsis* and its relation to tocopherol biosynthesis. Plant Physiol. Biochem. 40 (11), 913-920.

Zarhloul, M.K., W. Friedt, M.R. Khoschkhoi Yazdi, and W. Lühs, 1999: Genetic transformation and shoot regeneration ability of resynthesised *Brassica napus* line 'RS 306'. Cruciferae Newsl. 21, 59-60.