Characterisation of plant tocopherol cyclases and their overexpression in transgenic *Brassica napus* seeds

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Abstract Tocopherols, collectively known as vitamin E, are only synthesised in photosynthetic organisms. Tocopherol cyclase (TC) catalyses the formation of the chromanol headgroup of the various tocopherol isomers. TCs from Arabidopsis and maize (*Zea mays*) were expressed in *Escherichia coli* and purified. Analysis of the enzymatic properties revealed similarities but also differences between the two enzymes. Overexpression of chimeric TC gene constructs in developing seeds of transgenic rapeseed plants enhanced and modified the relative abundance of individual tocopheranol species in the seed oil, indicating a regulatory function of the enzyme in prenyllipid metabolism. © 2005 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

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1. Introduction

Tocopherols are synthesised in higher plants and other oxygenic photosynthetic organisms such as cyanobacteria [1] and are essential components of animal diets, therefore collectively known as vitamin E [2,3]. Tocopherols consist of a polar chromanol ring with a lipophilic prenyl side chain and comprise four homologous forms, namely \(\alpha\)-, \(\beta\)-, \(\gamma\)- and \(\delta\)-tocopherol, differing only in the number and position of methyl substituents on the chromanol head group [3]. These different tocopherol forms have varying antioxidative abilities, with \(\alpha\)-tocopherol being the most biologically active. Besides this well characterised function as lipid-soluble antioxidants that are tocopherol being the most biologically active. Besides this well characterised function as lipid-soluble antioxidants that are hydrolysis and have a role in the regulation of transcription and post-translational processes [4,7–11].

The main features of the biosynthetic pathway of tocopherols in plants have been elucidated several years ago using classical biochemical methods [12,13]. The identification of the involved genes during the past few years now facilitates the detailed characterisation of the pathway and permits new insights into the regulatory relationships among the respective enzyme activities [14–19]. Tocopherol biosynthesis proceeds at the inner envelope membrane of chloroplasts [20,20–22] utilising homogentisate derived from the cytosolic shikimate pathway [14,23,24] and phytid diphosphate, an isoprene intermediate from the plastidial 1-deoxyxylulose-5-phosphate pathway [25–28]. This first committed step is catalysed by the homogentisate pyrrolyltransferase producing 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) [15,18,29]. Introduction of another methyl group at position 3 of the chromanol ring by an MPBQ methyltransferase yields 2,3-dimethyl-5-phytyl-1,4-benzoquinol (DMPBQ) [30]. As shown in Fig. 1, at this point the pathway branches. On the one hand, cyclisation and methylation transform DMPBQ to \(\gamma\)- and \(\alpha\)-tocopherol, respectively, principally occurring in plants. On the other hand, MPBQ can be cyclised to \(\delta\)-tocopherol and subsequently methylated to \(\beta\)-tocopherol, which represent minor tocopherol components in plants [5,17,22,31–33]. The cyclisation reaction with DMPBQ or MPBQ as substrate is catalysed by tocopherol cyclase (TC). This enzyme forms the chromanol ring structure of the tocopherol, which is essential for the antioxidative radical scavenging function, by generating an additional oxygen heterocycle next to the aromatic ring originating from homogentisate (Fig. 1) [20,22,32,34–37].

So far, TC has been purified and characterised only from the cyanobacterium *Anabaena variabilis* [32,36]. Recently, however, the genes coding for the TC from *Synechocystis* sp. PCC6803, *Arabidopsis thaliana* and *Zea mays* have been cloned by Porfirova et al. [17] and Sattler et al. [38] as well as by our group, providing new opportunities to characterise TC and to investigate the importance of this enzymatic activity in controlling tocopherol biosynthetic rates.

In this paper, we report the purification of the TCs from *A. thaliana* and *Z. mays* via overexpression as recombinant proteins in *Escherichia coli* and discuss the differences in the enzymatic properties of the cyclases from these two plant species. In addition, we show that overexpression of chimeric TC...
genes in developing seeds of transgenic rapeseed plants can increase the total content and change the composition of tocopherols in the seed oil.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli strains, XL1 blue and DH5α, were used for cloning and propagation of recombinant plasmids. E. coli BL21staur (DE3) was used for expression of recombinant proteins. Transformed cells [39] carrying expression plasmids were grown at 37 °C in TB medium supplemented with 10 g/l of kanamycin and 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h at 22 °C. After induction, the cells were harvested by centrifugation, washed in 50 mM sodium phosphate buffer, pH 8.0, and used for protein purification and enzyme assays.

2.2. Development of TC expression vectors

Total RNA was extracted from leaves of A. thaliana ecotype Columbia and Z. mays cv. Magister using a LiCl-RNA isolation method [40]. Oligo-dT-Dynabeads (Dynal Biotech GmbH, Hamburg, Germany) were used for mRNA isolation and first strand cDNA synthesis was carried out with MMLV reverse transcriptase (Promega, Germany) for mRNA isolation and first strand cDNA synthesis was carried out with MMLV reverse transcriptase (Promega, Germany) and the cDNA synthesis was carried out with MMLV reverse transcriptase (Promega, Germany) were used for expression plasmids. Transformed cells [39] carrying expression plasmids were grown at 37 °C in TB medium supplemented with 10 g/l of kanamycin and 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h at 22 °C. After induction, the cells were harvested by centrifugation, washed in 50 mM sodium phosphate buffer, pH 8.0, and used for protein purification and enzyme assays.

2.3. Purification of recombinant cyclase proteins from A. thaliana and Z. mays

The pellets of the cells expressing the recombinant TC proteins were resuspended in 50 ml lysis buffer per 1 l cell culture (50 mM sodium phosphate, 2 mM PMSF, 1 mM DTT, and 10 mM imidazole, pH 8.0). Disruption of the cells was achieved by addition of lysozyme to a final concentration of 1 mg ml⁻¹ on ice for 30 min followed by sonication on ice twice for 30 s. DNasel and RNasel (final concentration 10 μg ml⁻¹) were added to the cell lysate and incubated on ice for 15 min. After cell debris were sedimented by centrifugation at 12000 × g for 20 min at 4 °C, soluble proteins were separated from the membranes by high-speed centrifugation at 15000 × g for 1 h, 4 °C and subsequently incubated with 300 μl Ni-NTA matrix (Qiagen, Hilden, Germany) per 1 l culture at 1 h at 4 °C with constant shaking. The suspension was then poured into the column. The column was washed with 10 column volumes of wash buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, and 20 mM imidazole) and His-tagged TC proteins were finally eluted with elution buffer (wash buffer containing 250 mM imidazole). Purified TC fractions were concentrated and dialysed buffer was exchanged against storage buffer (10 mM KH₂PO₄, 200 mM MgCl₂, 7 mM β-mercaptoethanol, 50% (v/v) glycerol, and 1 mM EDTA) using Vivaspin tubes (Vivascience, Hanover, Germany). The purified fractions were frozen in liquid nitrogen and stored at −80 °C. Protein concentration was estimated by Bradford protein assays, using bovine serum albumin as standard. Standard methods were used for SDS-PAGE analysis [42]. For Western blot analysis, proteins were transferred to PVDF membranes by semi-dry blotting and the His-tagged recombinant cyclase proteins were detected by chemiluminescence (LAS-1000, Raytest, Straubenhardt, Germany) using penta-His antibodies from mice (Qiagen, Hilden, Germany) and goat anti-mouse IgG-POD conjugate antibodies (Qiagen, Hilden, Germany) with Lumi-Light plus (Roche, Mannheim, Germany) as substrate. Pre-stained and His-tagged (Best Of All) protein markers were obtained from New England Biolabs (Beverly, MA, USA) and Biomol (Plymouth Meeting, PA, USA).

2.4. Molecular mass determination

The native molecular mass of the purified TC from A. thaliana was determined by gel filtration chromatography with the Duobios FPLC system (BioRad, München, Germany) using a guard column (Pharmacia P30-3, 300 mm × 1.5 cm) and a molecular mass standard kit (BioRad, München, Germany). The column was eluted with 100 mM NaH₂PO₄, 150 mM NaCl buffer, pH 8.0, at a flow rate of 1 ml min⁻¹ and calibrated with a protein standard kit (BioRad, München, Germany).

2.5. Synthesis and formulation of the substrates MPBQ and DMPBQ

The substrates MPBQ and DMPBQ were synthesised according to the protocol of Soll [29]. Freshly distilled BF₃-etherate (0.3 ml in 1 ml tetrahydrofuran) was added dropwise to a solution of 1.1 mmol quinol, 200 mg Al₂O₃ (W-200 basic) and 1.1 mmol isophytol in 2 ml dry tetrahydrofuran. The mixture was stirred under argon in the dark for 35 hrs. Residual BF₃ was hydrolysed on ice, and the prenylated quinols extracted with diethyl ether, the ether solution dried, and the organic solvent evaporated. The non-polar impurities were separated by column chromatography (silica 60, diethyl ether/pentane, 1:5) and the product was eluted with diethyl ether/pentane (1:2). The isomeric mixture of product quinols was separated by preparative HPLC (LiChrosorb, diethyl ether/pentane, 1:2). The identity of both substrates was verified by NMR spectroscopy, GC-MS and HPLC.

The substrates of the TC are hydrophobic and have a low solubility in aqueous assay buffer, so that the formulation of the lipophilic substrate in a water-soluble methyl-β-cyclodextrin complex is an indispensable necessity [36]. Therefore, MPBQ and DMPBQ were formulated with methyl-β-cyclodextrin in a similar way as outlined by Stocker et al. [36]. Briefly, 2 mg (4.8 μmol) of MPBQ and DMPBQ, respectively, was dissolved in 1 ml of 45.5 mM cyclodextrin solution
in 50 mM potassium phosphate, pH 7.0, by stirring for 15 min at RT. Subsequently, the formulated substrates were reduced by the addition of the same volume of 500 mM ascorbic acid in 50 mM potassium phosphate, pH 7.0, and a 15 min incubation at 30 °C with constant shaking. The formulated substrate was stored at −20 °C.

2.6. TC assays
TC activity was routinely determined in a reaction mixture composed of 200 mM potassium phosphate, pH 7.3, 4 mM dithiothreitol (DTT), 75 mM ascorbic acid, and 180 mM formulated DMPBQ using 250 ng to 50 μg of recombinant cyclase proteins in 100 μl reaction volume. After incubation at 30 °C for 30 min, the assay was stopped with 200 μl ethanol containing 80 ng α-tocopherol as internal standard and extracted in 1 ml hexane. The reaction products were separated from the substrates by normal-phase HPLC (Agilent 1100 series, Agilent, Böblingen, Germany) on a EC 250/4 Nucleosil 100–5 column (Macherey–Nagel, Düren, Germany) with an isocratic solvent system of 0.5% iso-propyl alcohol in heptane (HPLC grade, Roth, Germany) at a flow rate of 1.5 ml min⁻¹. Tocopherols were detected and quantified using a fluorescence detector set at 295 nm excitation and 320 nm emission wavelength.

2.7. Development and analysis of transgenic Brassica napus plants
Hypocotyl segments of spring rapeseed (cv. Drakkar) were transformed with Agrobacterium tumefaciens C58C1 AThV cells harbouring pATTC or pZmTC and regenerated to intact plants using a protocol as described earlier [43]. Successful gene transfer was initially confirmed by NPTII ELISA assays (Aqua Inc., Elkhart, Indiana, USA) of T1 plantlets. Transgenic plants of the T1 and T2 generation were grown under temporally and spatially different environments (average temperature 15 °C, 16 h day light) in the greenhouse in order to increase seed material and to confirm the transgenic phenotype in the seed progeny in comparison to respective control plants (cv. Drakkar).

To gain access to the TC genes and proteins, at first we cloned the respective gene from Synechocystis [46]. This was achieved by generating Synechocystis disruption mutants of several candidate genes by homologous recombination and subsequent analysis of the tocopherol composition by HPLC. These experiments resulted in the identification of the slr1737 gene encoding TC [46]. As reported by Provencher et al. [47], the deduced amino acid sequence showed significant similarity to the maize protein encoded by the sucrose export defective 1 (SXYD1, GenBank Accession No. AF302187) gene as well as the respective one from Arabidopsis encoded by the At4g32770 gene (GenBank Accession No. AL022537). The maize and Arabidopsis proteins have lengths of 474 and 488 amino acids, respectively, with predicted cleavable transit peptides of 65 and 76 amino acids, respectively, for the import into plastids [47].

To provide definite evidence that the proteins encoded by the Arabidopsis At4g32770 and the maize SXYD1 gene are functional TCs, the corresponding cDNAs lacking the predicted plastidial targeting sequences [47] were used for expression studies in E. coli by means of in vitro enzyme assays. For these assays, the lipophilic substrate 2,3-dimethyl-5-phytly-1,4-benzoquinol (DMPBQ) was synthesised [29] and converted into a water-soluble inclusion complex with cyclodextrin as described by Stocker et al. [37]. Subsequently, the cyclisation product γ-tocopherol was separated from the substrate by HPLC and quantified using α-tocopherol as an internal standard (Fig. 2). TC proteins from both Arabidopsis and maize were expressed in E. coli and showed high cyclase activity in the assays, thereby confirming the recently published data [17,38] that the corresponding genes from A. thaliana and Z. mays code for TCs.

As exemplified in Fig. 2 for the recombinant protein from A. thaliana, assays with soluble and membrane fractions of the E. coli host cells expressing the recombinant TC proteins from Arabidopsis and maize showed that the cyclase activities were predominantly found in the soluble fraction. This subcellular localisation is consistent with the amino acid sequences of the proteins lacking obvious transmembrane domains (http://www.cbs.dtu.dk/services/TMHMM) [48]. In planta TC appears to be associated with the envelope membranes of plastids, where prenyltransferase and methyltransferases involved in tocopherol biosynthesis are located [22]. On the other hand, the TC from Anabaena variabilis behaves like an integral membrane protein, because solubilisation with detergents was required in the course of the purification procedure [32].

3.2. Purification of recombinant TC from Arabidopsis and maize
To analyse the properties of the TC from Arabidopsis and maize, we purified the recombinant proteins containing a C-terminal His-tag fusion from E. coli cell lysates via Ni–NTA affinity chromatography after optimising the expression conditions of the recombinant TC proteins in E. coli. In comparison with inductions of the gene expression in the exponential growth phase of the bacterial cultures, inductions at relatively high cell densities were found to improve the level of

![Fig. 2. TC activity in the soluble and membrane fraction of E. coli cells overexpressing the recombinant protein from Arabidopsis thaliana. Separation and quantification of substrate (DMPQ) and reaction product (γ-tocopherol) of the cyclase assays were carried out via HPLC as described in 2 (IS, internal standard α-tocopherol; γ-Toc, γ-Tocopherol; DMPQ, 2,3-dimethyl-5-phytlybenzoquinol; mV, fluorescence signal).](image-url)
recombinant TC proteins appreciably within the E. coli cells, especially in the case of the maize protein. The purification of the recombinant enzymes during the consecutive stages of chromatography was monitored by enzymic assays as well as SDS gel electrophoresis and Western blot analysis (Fig. 3A).

According to the protein pattern in SDS/PAGE analyses, a significant enrichment could be achieved for both recombinant TCs after elution from the affinity matrix (Fig. 3, lane El). Additional gel filtration of the recombinant TC resulted in no further purification effect (Fig. 3B), but could be used to verify the molecular mass of the catalytically active cyclase under non-denaturing conditions. Fig. 4 shows the elution profile of the Arabidopsis TC from the gel filtration column. The cyclase activity eluted with a molecular mass of about 50 kDa, corresponding to the calculated molecular mass of 47 kDa (Fig. 4). These data show that the mature cyclase protein is active in its monomeric state, and does not require multimeric aggregates in contrast to the cyclase involved in β-carotene biosynthesis [49], at least under in vitro conditions.

Attempts to purify the recombinant TC to homogeneity for instance by ion exchange chromatography or hydrophobic interaction chromatography were of no avail, since the enzymatic activity was always lost during such subsequent purification steps. But altogether the enriched Arabidopsis and maize TC fractions obtained after affinity chromatography were sufficiently pure and stable to allow enzymatic characterisation.

3.3. Enzymatic characterisation of the recombinant TCs

In order to analyse the properties of the recombinant TC from Arabidopsis and maize, we optimised the in vitro enzyme assay conditions using the enriched Arabidopsis and maize TC protein fractions obtained after Ni–NTA affinity chromatography. To quantitatively convert hydrophobic substrates, DMPBQ, into a water-soluble methyl-β-cyclodextrin complex, we tried different proportions of substrate and cyclodextrin. A molar ratio of 1:20 between substrate and the complex forming reagent turned out to yield an almost complete recovery of the substrates (≥ 90%) in the water-soluble complex and highest cyclase activities. Furthermore, the redox state, from both, the substrate as well as the catalytic reaction centre of the cyclase, are vitally important for optimal turnover rates [22,36].

We figured out that the addition of dithiothreitol and ascorbate to a final concentration of 4 and 75 mM, respectively, gave maximal cyclase activities. Besides, divalent cations like Mg²⁺, for instance, are not imperative for cyclase activity, as depletion of divalent cations from the cyclase assay had no effect on the activity of TC from both Arabidopsis and maize.

The pH dependence of both cyclases was then analysed in a buffer system covering the range of pH 5.5–9.0. As shown in Fig. 5A, the recombinant TC from A. thaliana had a pH-optimum of pH 7.0, whereas the maize enzyme possessed a broader pH-optimum between pH 7.5 and 9.0. Further analysis testing different buffer components and concentrations revealed that the cyclases from both Arabidopsis and maize were most active in 200 mM phosphate buffer.

The optimal assay temperature also differed clearly between the two cyclases (Fig. 5B). The Arabidopsis enzyme showed highest activities at 30°C, whereas the maize cyclase activity...
reached a maximum at 40°C. As depicted in Fig. 6, both Arabidopsis and maize TC showed highest activities in the presence of about 350 mM DMPBQ, with a slightly higher \( K_m \) value of 180 \( \mu \)M for the maize TC, compared to a \( K_m \) value of 90 \( \mu \)M for the Arabidopsis one.

In summary, in spite of the high sequence similarities between the TC from Arabidopsis and maize, the enzymes differ in their properties. These differences are likely due to the diverse origins of the assayed enzymes because we compare the properties of a protein from a C3 dicot plant with those from a C4 monocot one, therefore meeting other demands concerning adaptation to environmental conditions.

As a next step, we analysed the activity of the recombinant TC from Arabidopsis and maize with regard to the substrates MBPQ and DMPBQ. TC assays using various concentrations of formulated MBPQ were conducted, but even under conditions under which DMPBQ was almost completely converted to \( \gamma \)-tocopherol, no formation of the respective cyclisation product \( \delta \)-tocopherol could be detected by HPLC analysis (Fig. 7C), independent of the presence or absence of a C-terminal fused His-Tag. Stocker et al. [32] provided conclusive evidence that the E-configuration of the double bond in the precursor and the chirality in the phytyl substructure are, for instance, critical determinants for the cyclisation reaction. To exclude that erroneously an inoperative stereoisomer of MPBQ was selected during the chemical synthesis of the substrates, we confirmed the fidelity of MPBQ by mass spectroscopy and NMR. Hence, these findings suggest that the recombinant Arabidopsis and maize cyclase activities differ, at least under in vitro conditions, from the previously reported data obtained with the purified TC from \textit{A. variabilis} [32], which shows no pronounced specificity with regard to degree and position of methylation at the aromatic ring of the substrate. Porfirova et al. [17] and Cheng et al. [50] analysed the tocopherol compositions of Arabidopsis mutants deficient in either TC [17] or MPBQ methyltransferase activity [40] and provided strong evidence that the Arabidopsis TC is encoded by a single copy gene and can utilise not only DMPBQ but also MPBQ as substrate \textit{in planta}. The tocopherol isomers derived from MBPQ, namely \( \delta \)- and \( \beta \)-tocopherol, are only minor components in leaves and seeds of wild-type Arabidopsis plants. On the one hand, this can be due to a high metabolic methylation rate of MPBQ to DMPBQ by the MBPQ methyltransferase, thus depriving MPBQ as substrate from the cyclase. On the other hand, the marginal \( \delta \)- and \( \beta \)-tocopherol content can originate from a low affinity of the TC towards MBPQ. Taking these options into consideration, our in vitro conditions optimised with DMPBQ as substrate are obviously not suited for MBPQ conversion, suggesting that under these conditions affinity of the TC for MBPQ is too low or that other requirements like membrane or protein association or posttranslational modifications of TC, for instance, are mandatory in order to obtain a conversion of MBPQ by a plant TC.
3.4. Overexpression of chimeric TC gene constructs

In order to elucidate the relevance of the TC within the course of the tocopherol biosynthesis and its impact on the tocopherol quantity, we produced transgenic *Brassica napus* plants overexpressing the TCs from Arabidopsis and maize, respectively. For this purpose, we developed binary plant transformation vectors harbouring the NptII gene conferring kanamycin resistance and the TC coding sequence from Arabidopsis or maize under the control of the strong seed specific napin promoter. Transgenic *B. napus* lines were generated by *A. tumefaciens* mediated transformation [43]. Seed oil analyses of NPTII-ELISA positive transgenic *B. napus* plants and their progeny, with regard to tocochromanol composition and content, were carried out via HPLC. The results for the respective T1 and T2 populations and selected individual plants are shown in Table 1. In comparison to the control plants, the average total tocochromanol content in the seed oil of the T1 populations expressing the maize or Arabidopsis TC was significantly increased by 18% and 28%, respectively. Analyses of the T2 population of selected T1 plants verified this enhancement in total tocochromanol content both on an average and individual plant level. For example, an increase of 20% to 55% was observed in the seed oil of selected T2 plants as compared to the corresponding wild-type plants (Table 1). Similar results have been obtained by our group (Sadre et al., in preparation) and Lassner et al. [51] with transgenic *B. napus* plants expressing the homogentisate phytlytransferase from Arabidopsis. This enzyme catalyses the formation of MPBQ and overexpression causes a comparable increase in substrate flux and therefore in total tocopherol quantity in transgenic *B. napus* seeds. In conclusion, we can ascertain that TC influences to a certain extent total tocopherol content in plants by effectively channelling the flux of the prenylquinone intermediates towards the different tocopherol end products.

In addition, we observed interesting differences concerning the tocochromanol composition of the seed oil between wild-type and transgenic *B. napus* plants overexpressing the TCs from Arabidopsis or maize (Table 1). The average δ-tocopherol content in the transgenic T1 population expressing the maize TC showed a slight increase of 1.6-fold compared to the wild-type and a significant one of 2.7-fold in the transgenic plants expressing the Arabidopsis TC. Analyses of the T2 populations of selected T1 plants confirmed these results, in fact we found a significant 1.8- and 3.6-fold enhancement for the plants expressing the maize or Arabidopsis TC, respectively. In comparison, to the control plants the δ-tocopherol content of individual T2 plants even showed a 3.2-fold (ZmTC/677-36) increase.

Table 1

<table>
<thead>
<tr>
<th>Tocochromanol content (mg kg⁻¹ oil)</th>
<th>NacdP8 Total</th>
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<tbody>
<tr>
<td><strong>Wild-type</strong></td>
<td></td>
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<tr>
<td>ZmTC</td>
<td>25</td>
</tr>
<tr>
<td>AtTC</td>
<td>36</td>
</tr>
<tr>
<td><strong>Selected T1 plants</strong></td>
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<tr>
<td>ZmTC/677</td>
<td>1</td>
</tr>
<tr>
<td>AtTC/684</td>
<td>1</td>
</tr>
<tr>
<td><strong>Wild-type</strong></td>
<td></td>
</tr>
<tr>
<td>ZmTC/677</td>
<td>17</td>
</tr>
<tr>
<td>AtTC/684</td>
<td>40</td>
</tr>
<tr>
<td><strong>Selected T2 plants</strong></td>
<td></td>
</tr>
<tr>
<td>ZmTC/677-36</td>
<td>1</td>
</tr>
<tr>
<td>ZmTC/677-5</td>
<td>1</td>
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<tr>
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</tr>
<tr>
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<td>1</td>
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<tr>
<td>AtTC/684-37</td>
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</tbody>
</table>

Mean values ± SE and individual values of selected plants are shown (N, number of analysed transgenic and control plants, respectively; ZmTC, AtTC, and transgenic *Brassica napus* populations expressing the TC from maize and Arabidopsis, respectively). T1 and T2 plants were grown in different environments, hence the absolute values for the respective wild-type plants differed.

*P < 0.01, significant.*
and 6.7-fold (AtTC/684-5) increase (Table 1). These alterations directly attribute to the activity of the recombinant TCs in the respective transgenic plants, since transgenic *B. napus* lines expressing the homogentisate phytyltransferase though showing an increase in total tocopherols had no elevated δ-tocopherol levels (Sadre et al., in preparation). In concordance with the analysis of the Arabidopsis mutants [17,50], the TCs from Arabidopsis and maize are able in planta to utilise not only DMBPQ but also MPBQ as a substrate, at least under conditions that provide elevated MPBQ concentrations or TC expression levels. As discussed above, so far these findings are not supported by the results of the TC assays in vitro.

Furthermore, transgenic rapeseed plants expressing the TC from Arabidopsis or maize showed a noticeable variance regarding the P8 content (Table 1). P8 is a minor tocochromanol component in most seed oils and predominantly found in the oil of *B. napus, Linum usitatissimum* and *Cannabis sativa* seeds [45,52–56]. In the transgenic plants expressing the maize TC, we detected a slight rise of the average P8 content, whereas the transgenic plants expressing the Arabidopsis TC showed a significant 1.9 to 2.4-fold enhancement in the T1 and T2 population, respectively. Analyses of individual T2 plants confirmed the tendency for the maize progeny and substantiated the results for the Arabidopsis plants by reaching a maximum of 7.6-fold increase (AtTC/684-16) in the P8 content compared to the wild-type plants (Table 1). In conclusion, our findings provided the indication that plant TCs, especially from Arabidopsis, possess a broader substrate specificity and also accept 2,3-dimethyl-5-solanesyl-1,4-benzoquinol (DMSBQ, Plastoquinone-9) as a substrate (Fig. 8).

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**References**


