of 15 min until fusion occurred. Fused oocytes were again electrically stimulated (20-v/mm dc pulses for 20 μs) to ensure activation. Nuclear transplant oocytes were immediately treated with cyclohexamide (10 μg/ml) in CR1-aa medium [C. F. Rosenkraus and N. L. First, *Theriogenology* **35**, 266 (abstr.) (1991)] with 3 mg of bovine serum albumin (fatty acid free) for 5 to 6 hours. After treatment, the oocytes were cultured in cyclohexamide-free medium. On day 3 (day 1 being the day of nuclear transfer), the nuclear transplant embryos were transferred to dishes containing CR-1aa medium supplemented with 10% FBS and mouse fetal fibroblast cells pretreated with mitomycin C (10 μg/ml) for 2.5 hours. On days 8 and 9 of in vitro culture, visually normal blastocysts were selected and transferred to recipient cows.

13. Genomes of recipient cows, nuclear donor cells, and cloned calves were typed for microsatellites by means of 23 primer sets that were provided by Shirakawa Institute of Animal Genetics, Livestock Technology Association of Japan [M. M. Inoue *et al.*, *Anim. Sci. Technol.* **68**, 443 (1997)].
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Elevating the Vitamin E Content of Plants Through Metabolic Engineering

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α-Tocopherol (vitamin E) is a lipid-soluble antioxidant synthesized only by photosynthetic organisms. α-Tocopherol is an essential component of mammalian diets, and intakes in excess of the U.S. recommended daily allowance are correlated with decreased incidence of a number of degenerative human diseases. Plant oils, the main dietary source of tocopherols, typically contain α-tocopherol as a minor component and high levels of its biosynthetic precursor, γ-tocopherol. A genomics-based approach was used to clone the final enzyme in α-tocopherol synthesis, γ-tocopherol methyltransferase. Overexpression of γ-tocopherol methyltransferase in *Arabidopsis* seeds shifted oil compositions in favor of α-tocopherol. Similar increases in agricultural oil crops would increase vitamin E levels in the average U.S. diet.

The chloroplasts of higher plants produce numerous compounds that not only perform vital functions but also are important from agricultural and nutritional perspectives. Tocopherols, the lipid-soluble antioxidants known collectively as vitamin E, are one such group of compounds. The four naturally occurring tocopherols, α-, β-, γ- and δ-tocopherol, differ only in the number and position of methyl substituents on the aromatic ring (*1*). In addition to their role as antioxidants (*1*), tocopherols stabilize polyunsaturated fatty acids within lipid bilayers by protecting them from lipoxygenase attack (*2*).

Of tocopherol species present in foods, α-tocopherol is the most important to human health, has the highest vitamin E activity (*3*), and occurs as a single (*R,R,R*)-α-tocopherol isomer (*4*). Although all tocopherols are absorbed equally during digestion, only (*R,R,R*)-α-tocopherol is preferentially retained and distributed throughout the body (*5*).

The most recent U.S. recommended daily allowance (RDA) suggests that 10 to 13.4 international units (IU) of vitamin E [equal to 7 to 9 mg of (*R,R,R*)-α-tocopherol] be consumed daily (*6*). Because of the abundance of plant-derived components in most diets, this RDA is often met in the average diet. However, daily intake of vitamin E in excess of the RDA (100 to 1000 IU) is associated with decreased risk of cardiovascular disease and some cancers, improved immune function, and slowing of the progression of a number of degenerative human conditions (*5*). Obtaining these therapeutic levels of vitamin E from the average diet is nearly impossible unless a concerted effort is made to ingest large quantities of specific foods enriched in vitamin E.

In the United States, approximately 60% of dietary vitamin E intake is from vegetable oils (*7*). In soybean oil, which accounts for 80 and 25% of the edible oil consumed in the United States and the world, respectively (*8*), α-tocopherol and its immediate biosynthetic precursor γ-tocopherol account for 7 and 70%, respectively, of the total tocopherol pool (*9*). The other major oilseed crops—corn, canola, cottonseed, and palm oils—have similarly low α- to γ-tocopherol ratios (*4*).

Substantial increases in the α-tocopherol content of major food crops are needed to provide the public with dietary sources of vitamin E that can approach the desired therapeutic levels. The observation that many oilseeds contain relatively high levels of γ-tocopherol, the biosynthetic precursor to α-tocopherol, suggests that the final step of the α-tocopherol biosynthetic pathway, catalyzed by γ-tocopherol methyltransferase (γ-TMT), is limiting in these tissues. Therefore, it may be possible to convert the large pool of γ-tocopherol present in seeds such as soybeans to α-tocopherol by targeted overexpression of

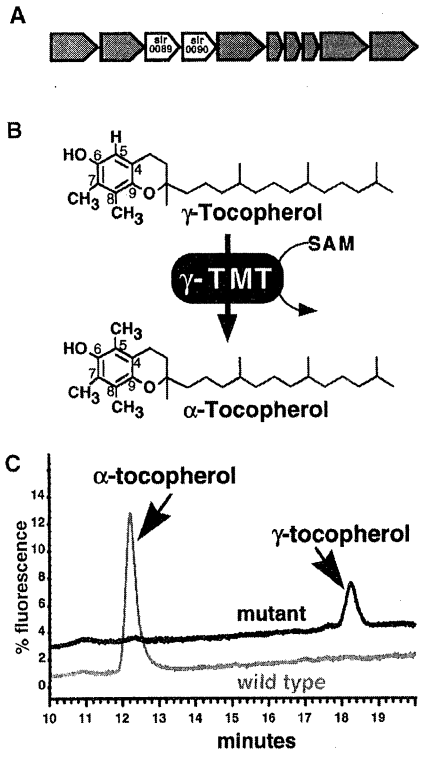

Fig. 1. γ-TMT in *Synechocystis* PCC6803. (**A**) Putative tocopherol biosynthetic operon from *Synechocystis* (*15*). SLR0089 encodes γ-TMT and SLR0090 encodes *p*-hydroxyphenylpyruvate dioxygenase. (**B**) γ-TMT enzymatic reaction. γ-TMT adds a methyl group to ring carbon 5 of γ-tocopherol. (**C**) HPLC profiles of tocopherols in wild-type *Synechocystis* PCC6803 and the γ-TMT null mutant. Total lipid extracts were isolated from each line, and tocopherols were analyzed by HPLC (*21*).

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the γ-TMT gene.

The α-tocopherol biosynthetic pathway is known (*10*), and genes encoding one enzyme, *p*-hydroxyphenylpyruvate dioxygenase (HPPDase) have been cloned from carrot (*11*) and *Arabidopsis* (*12*). It has proven difficult to obtain gene probes for additional pathway enzymes because the remaining enzymes are membrane-bound. We used a genomics-based approach to clone the γ-TMT gene that exploits the complementary model photosynthetic organisms *Arabidopsis thaliana* and *Synechocystis* PCC6803. Both organisms synthesize and accumulate (*R,R,R*)-α-tocopherol (*13*) to greater than 95% of their tocopherol pools, which suggests that γ-TMT activity is not limiting in these photosynthetic cells. We used the gene encoding the *Arabidopsis* HPPDase (*14*) to search the *Synechocystis* PCC6803 genomic database (*15*) and identified a single open reading frame (ORF) that shared 35% amino acid sequence identity with the *Arabidopsis* HPPDase. The *Synechocystis* HPPDase gene was located within a predicted 10-gene operon encompassing bases 2,893,184 to 2,905,235 of the *Synechocystis* genome (Fig. 1A). Because related biosynthetic genes in bacteria are often organized into operons, we hypothesized that this operon might contain other genes involved in tocopherol biosynthesis, including γ-TMT.

The operon included one candidate for a *Synechocystis* γ-TMT gene, ORF SLR0089. This ORF predicted a protein that shared a high degree of amino acid sequence similarity with known plant Δ-(24)-sterol-C-methyltransferases. SLR0089 also contains several structural features of a γ-TMT (Fig. 2) including *S*-adenosylmethionine (SAM)–binding domains (*16*) and a putative NH_2-terminal bacterial signal sequence (*17*), which is consistent with the presumed site of cyanobacterial tocopherol biosynthesis. The predicted 32,766-dalton mature SLR0089 protein is similar in size to the 33,000 daltons reported for the only γ-TMT purified from plants (*18*). We therefore hypothesized that the SLR0089 ORF encoded the *Synechocystis* γ-TMT.

A null mutant for the SLR0089 gene was created by replacing the wild-type gene with a nonfunctional SLR0089 gene (*19*) through homologous recombination (*20*). The SLR0089 mutant was then analyzed by high-performance liquid chromatography (HPLC) (*21*) for alterations in the normal *Synechocystis* tocopherol profile. Both the amount and type of tocopherol were altered. The mutant was devoid of α-tocopherol, accumulated the biosynthetic precursor γ-tocopherol, and produced less total tocopherol (Fig. 1C). The change in the tocopherol composition of the SLR0089 mutant is consistent with the phenotype expected for a mutant lacking γ-TMT activity. To confirm its identity, the SLR0089 ORF was expressed in *Escherichia coli* (*22*), and the recombinant protein was assayed for γ-TMT (*18*). The results showed that the recombinant SLR0089 protein was able to catalyze the methylation of γ-tocopherol to form α-tocopherol (Table 1). Thus, the SLR0089 ORF encoded the *Synechocystis* γ-TMT (Syn.γ-TMT).

To identify a γ-TMT gene from higher plants, we used the Syn.γ-TMT protein sequence as a query in a search of the *Arabidopsis* expressed sequence tag (EST) database (*23*). One cDNA clone, *Arabidopsis* EST 165H5T7, showed 66% amino acid sequence similarity with Syn.γ-TMT (Fig. 2). The 165H5T7 protein contained structural features common to γ-TMTs (Fig. 2), including SAM binding domains and a predicted 47–amino acid chloroplast transit peptide (*24*), which is consistent with the known intracellular locale of tocopherol synthesis in plants (*25*). The mature 33,843-dalton 165H5T7 protein is similar in size to the deduced mature Syn.γ-TMT protein. To confirm that 165H5T7 encoded an *Arabidopsis* γ-TMT ortholog, the DNA sequence corresponding to the predicted mature 165H5T7

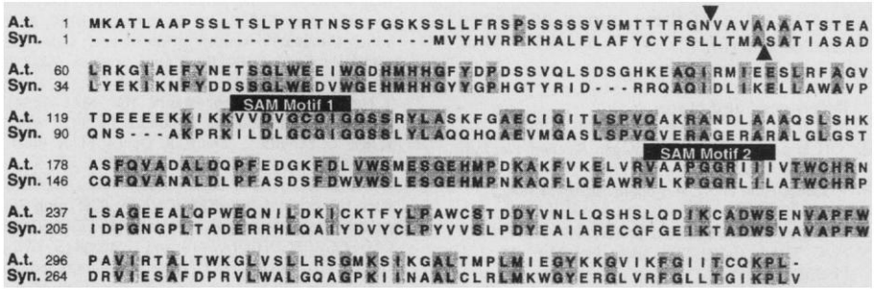

Fig. 2. Alignment of γ-TMT protein sequences from *Arabidopsis thaliana* (A.t.) (GenBank accession number AF104220) and *Synechocystis* PCC6803 (Syn.) (GenBank accession number D64004 AB001339). Residues that are identical in the two sequences are indicated by shading. Two motifs corresponding to conserved SAM binding domains (*16*) are indicated. Predicted cleavage sites of NH_2-terminal targeting domains (*17*, *24*) are indicated in each sequence by solid triangles.

Table 1. Substrate specificity of *Synechocystis* and *Arabidopsis* γ-TMTs. In vitro γ-TMT assays (*18*) were performed on extracts from *E. coli* expressing the predicted mature proteins of *Synechocystis* (GenBank accession number D64004 AB001339) and *Arabidopsis* γ-TMT (GenBank accession number AF104220) (*22*, *26*). The identification of assay products was confirmed by HPLC analysis (*21*). Values are represented as the mean ± SE of triplicate assays.

Substrate	Specific activity (nmol per mg of protein per hour)		Product
	Syn.γ-TMT	A.t.γ-TMT	
γ-Tocopherol	0.12 ± 0.01	3.57 ± 0.12	α-Tocopherol
β-Tocopherol	0	0	None
δ-Tocopherol	0.12 ± 0.01	1.32 ± 0.17	β-Tocopherol

Table 2. Tocopherol content and compositions of segregating T2 seed populations from *Arabidopsis* transformed with the empty vector control pDC3 and with the γ-TMT overexpression construct pDC3-A.t.γ-TMT (*29*). Tocopherol content and composition were determined by HPLC analysis (*21*) and are represented as the mean ± SE of four replicate seed samples from each transformed *Arabidopsis* line. The pDC3 results were determined from the pooled data obtained from the analysis of three independent transformed lines. γ-TMT activity (*18*) was determined in triplicate from mature seed extracts.

Line	Tocopherol content (ng per mg of seed)	α-Tocopherol (%)	β-Tocopherol (%)	γ-Tocopherol (%)	δ-Tocopherol (%)	γ-TMT activity (pmol per mg of protein per hour)
pDC3	366.3 ± 24.4	1.1 ± 0.4	0	96.9 ± 1.0	2.18 ± 0.61	0
pDC3-γ-TMT-1	360.6 ± 6.0	95.1 ± 0.6	1.0 ± 0.5	3.9 ± 0.6	0	2.12 ± 0.10
pDC3-γ-TMT-2	383.4 ± 22.5	93.5 ± 1.0	1.0 ± 0.2	5.5 ± 0.9	0	1.88 ± 0.06
pDC3-γ-TMT-3	339.7 ± 26.7	87.8 ± 1.1	0.5 ± 0.2	12.0 ± 0.9	0	1.71 ± 0.05

protein was expressed in *E. coli* (*26*). The recombinant 165H5T7 protein indeed possessed γ-TMT activity (Table 1) (*18*). Thus, the protein encoded by EST clone 165H5T7 was the *Arabidopsis* γ-TMT (A.t.γ-TMT).

The substrate specificities of the *Synechocystis* and *Arabidopsis* γ-TMTs (*18*, *27*) were similar. When different methyl-substituted tocopherols were used as substrates, only δ-tocopherol and γ-tocopherol were methylated by the two γ-TMTs to yield β-tocopherol and α-tocopherol, respectively (Table 1). Thus, the 5 position of the tocopherol aromatic head group is the specific site of methylation by γ-TMT.

To determine whether γ-TMT is a key enzymatic activity involved in determining the tocopherol composition in seeds, *Arabidopsis* was transformed (*28*) with pDC3-A.t.γ-TMT (*29*), a plant expression construct containing the full-length *Arabidopsis* γ-TMT cDNA driven by the seed-specific carrot DC3 promoter (*30*). *Arabidopsis* was used because, like major oilseed crops, its seed tocopherol profile (>95% γ-tocopherol) suggests that γ-TMT activity is limiting. Primary transformants were selected by antibiotic resistance and grown to maturity. Pooled, segregating T2 seeds from individuals were analyzed for changes in tocopherol content and composition.

HPLC (*21*) analysis showed that seed-specific overexpression of the *Arabidopsis* γ-TMT gene increased seed α-tocopherol levels >80-fold (Table 2). Seeds of the lines overexpressing the largest amounts of γ-TMT contained >95% of their total tocopherol pool as α-tocopherol (Table 2). In addition, the small δ-tocopherol pool normally present in wild-type *Arabidopsis* seed was completely converted to β-tocopherol, confirming the in vitro finding that both γ- and δ-tocopherols are substrates for *Arabidopsis* γ-TMT in vivo. The observed changes in the seed tocopherol composition of γ-TMT overexpressers correlated with increased levels of γ-TMT enzyme activity in mature seed protein (Table 2) (*18*). The accumulation of α-tocopherol and β-tocopherol in the seeds of plants overexpressing γ-TMT indicates that their low levels in wild-type *Arabidopsis* seed were due to limiting seed γ-TMT activity.

Overexpression of the γ-TMT gene altered the tocopherol composition of *Arabidopsis* seed but not the total tocopherol content (Table 2). Thus, the increased α-tocopherol content of pDC3-A.t.γ-TMT seeds was due to the conversion of the large pool of γ-tocopherol already present in wild-type seeds. Therefore, it appears that γ-TMT plays an important role in determining the composition but not the total content of the seed tocopherol pool.

The vitamin E activity of seeds from *Arabidopsis* lines overexpressing γ-TMT was ninefold that of wild-type seeds. Given the differing vitamin E potencies of α-, β-, γ- and δ-tocopherols [100, 50, 10, and 3% relative vitamin E activity, respectively (*3*)], 50 gm of wild-type *Arabidopsis* seed oil would contain 9 IU of vitamin E, whereas 50 g of seed oil from the best transgenic lines would contain 84 IU. We may assume that γ-TMT activity is also limiting in commercially important oilseeds crops that have low α- to γ-tocopherol ratios, such as soybean, corn, and canola. Overexpressing the γ-TMT gene in these crops should similarly elevate α-tocopherol levels and thereby increase the nutritional value of these important dietary sources of vitamin E.

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22. The amplified SLR0089 gene was subcloned into pET3D (Novagen, Madison, WI), generating plasmid pSyn.γ-TMT. Protein expression was induced as described [F.W. Studier *et al.*, *Methods Enzymol.* **185**, 60 (1990)], and extracts from induced pSyn.γ-TMT and pET3D empty vector control cells were prepared by homogenizing cell pellets in 10 mM Hepes (pH 7.8), 5 mM dithiothreitol, 0.24 M sorbitol, and 1 mM phenylmethylsulfonyl fluoride. Triton X-100 was added to a final concentration of 1%, and the homogenates were incubated on ice for 30 min and centrifuged at 30,000*g* for 30 min at 4[1/4]C, after which the protein concentration of each supernate was determined [M. Bradford, *Anal. Biochem.* **72,** 248 (1976)].
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27. Substrate specificity assays were performed as described (*18*), except that 5 mM of the appropriate tocopherol substrate was substituted for γ-tocopherol. Radiolabeled reaction products were then collected after normal phase HPLC fractionation (*21*), and radiolabel incorporation was determined by scintillation counting.
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