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Enhanced Biosynthesis of α-tocopherol in Transgenic Soybean by Introducing γ-TMT gene

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Abstract

This study was conducted to improve tocopherol (vitamin E) composition in soybean (*Glycine max*) by introducing a gamma-tocopherol methyl transferase (γ-TMT) gene via *Agrobacterium tumefaciens-mediated transformation*. Immature cotyledon explants were co-cultivated with *Agrobacterium tumefaciens*. Putative transgenic embryos were selected from immature cotyledons on MS medium supplemented with 40 mg/L 2,4-D containing 100 mg/L kanamycin, 500 mg/L carbenicillin and 250 mg/L cefotaxime. Plantlets were developed from somatic embryos, and then transferred to soil. Nineteen regenerated plantlets obtained on the selection medium from 1,460 cotyledons. However, only 9 plantlets were confirmed as transformed plants. Integration of the transgene into the soybean genomic DNA was confirmed by PCR and Southern blot analysis. HPLC analysis showed that the content of α-tocopherol in transgenic soybean seeds (AT-1) was approximately 4-fold higher than that of non-transgenic plants. Conclusively, we obtained the transgenic soybean having increased α-tocopherol content by the overexpression of γ-TMT transgene.

Key words: Soybean, *Agrobacterium*, transformation, somatic embryogenesis, HPLC, tocopherol, γ-TMT

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Introduction

Soybean is a major food source which is worldwide dicotyledonous crops. Soybean seeds contain about 40% protein and 20% oil. Moreover, soybean oil contains high levels of tocopherols, and it is an important source of vitamin E (tocopherol) in human diet (Traber and Sies 1996). Vitamin E is powerful antioxidant that helps protect cells from the harmful oxidative free radicals. Clinical evidences suggest that vitamin E decreases the risk of cardiovascular disease, cancer and Alzheimer's, promotes immune system and circulatory function, defense against light-induced eye and dermal pathologies, and prevents various chronic degenerative diseases and premature aging (Pryor 2000; WCRF 1997). The major tocopherols found in the seeds and oils are γ- and α-tocopherols. The *in vivo* antioxidant activity of α-tocopherol is higher than that of the other isoforms and vitamin E activity of α-tocopherol is about 10-fold higher than that of its precursor γ-tocopherol (Fukuzawa et al. 1982; Kamal-Eldin and Appelqvist 1996). However, the α- to γ-tocopherol ratios of soybean is low, indicating composition of tocopherols is nutritionally poor. Development of new varieties by the traditional breeding methods has been limited due to the narrow genetic base in domestic soybean varieties. The development of gene transfer techniques for plant species is of great interest and value to plant breeders because they can be used for the rapid and effective transfer of beneficial foreign genes to plants.

Recently, improvement of crop quality has been a major topic in plant breeding. Genetic transformation technique provides new tool for plant breeding. Vitamin E content in food crops and vegetables can be improved through the metabolic engineering of the tocopherol biosynthesis pathway (Van Eenennaam 2003). α-Tocopherol is produced from γ-tocopherol by the reaction of γ-tocopherol methyltransferase (γ-TMT) (Hirschberg 1999). In an attempt to increase α-tocopherol content in several crop plants through metabolic engineering of tocopherol biosynthesis, Shintani and DellaPenna (1998) cloned and characterized a γ-TMT gene from *Arabidopsis*, and overexpressed the gene in *Arabidopsis*. Yun et al. (2001) also isolated a γ-TMT clone from a green perilla (*Perilla frutescense*) seed cDNA library and confirmed its function with the protein expressed by the clone. They also obtained transgenic lettuce and rice stably converting the γ-tocopherol pool to α-tocopherol by overexpression of γ-TMT gene. In addition, they are characterizing genes for the key enzymes of the pathway to increase tocopherol pools in major food crops and vegetables through metabolic engineering of the pathway. In the near future, coordinate manipulation of genes involved in the pathway will allow qualitative and quantitative improvement of vitamin E activity of food crops and vegetables.

Agrobacterium-mediated transformation has been most widely used due to the stability of gene introduced by this method. However, the use of this procedure has been limited by the host- and tissue-specificity associated with this biological vector. In addition, the efficiencies of transformation have been very low. Trick and Finer (1998) developed a new improved technique called sonication-assisted *Agrobacterium*-mediated transformation (SAAT), which consists of subjecting the target tissue to ultrasound while immerged in an *Agrobacterium* suspension to enhance transformation.

The objective of this study is to develop transgenic soybean plants with high α-tocopherol content via overexpression of γ-TMT gene in soybean.

Materials and Methods

Plant material

Soybean (*Glycine max* cv. Pungsannamulkong and Alchankong) pods were collected at about 2 weeks after flowering when the immature cotyledons were 3-4 ㎜ long and surface-sterilized by immersion in 70% ethanol for 1 min followed by soaking in a 2% sodium hypochlorite (NaOCl) for 20 min. The pods were then rinsed three times in sterile distilled water. Immature seeds were aseptically removed from the pods and the end containing the embryonic axis was cut off and discarded. The two cotyledons were then removed from the seed coat and separated.

Vector construction

Arabidopsis γ-TMT cDNA clone was obtained from *Arabidopsis* stock center at Ohio State University (USA). The clone was subcloned into the binary vector pBI 121 with the kanamycin resistance selectable marker under the control of CaMV35S promoter (Fig. 1). The resulting recombinant vector pYB-TMT was introduced into *Agrobacterium tumefaciens* strain LBA 4404 (An et al. 1988), and the strain was used for soybean transformation.

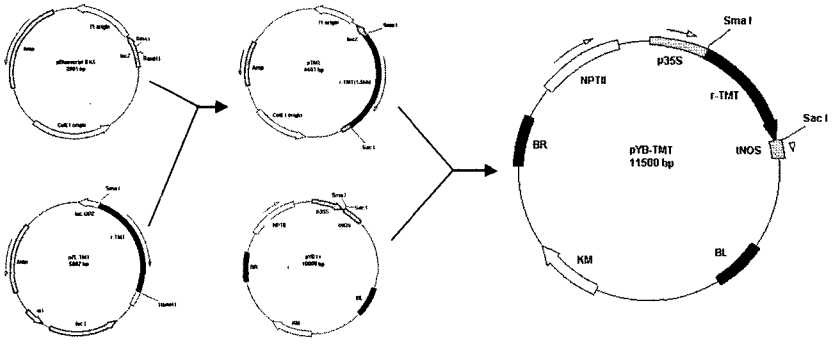

Figure 1. Structure of the γ-TMT recombinant vector pYB-TMT, which was constructed by ligation of γ-TMT sequence at the *Sma*I and *Sac*I sites of the binary vector pBI 121. Abbreviations used were: Pnos, nopaline synthase promoter; NPT Ⅱ, neomycin phosphotransferase gene Ⅱ; Tnos, polyadenylation signal of the nopaline synthase gene; p35S, 35S promoter of cauliflower mosaic virus (CaMV); γ-TMT, γ-tocopherol methytransferase; RB, T-DNA right border; LB, T-DNA left border.

Transformation

Agrobacterium was grown overnight in Luria-Bertani (LB) liquid medium (1% bacto-peptone, 0.5% bacto-yeast extract, 1% sodium chloride, pH 7.0) supplemented with 50 mg/L kanamycin with shaking at 27℃/150 rpm in the dark condition. The resulting culture was centrifuged at 3,000 rpm for 10 min to yield a bacterial pellet. The pellet was washed twice with equal volumes of liquid co-cultivation medium and resuspended as above to an OD_{600}=0.7 before use for the experiment. Twenty cotyledon explants were dipped into 50 ml Falcon tubes containing 20 ml of *Agrobacterium* suspension. Cotyledons in the tube were placed in a float at the center of a bath sonicator (Branson Co. USA) for 6 sec and were gently resuspended for 20 min. After sonication treatment, cotyledons were removed from the tubes, placed on sterile filter paper to blot off excess bacteria and placed adaxial side up on coculture medium [MS salts, 40 mg/L 2,4-D, 3% sucrose,

pH 7.5, 0.2% Gelrite, 100 μM acetosyringone (Aldrich, USA)] for 3 days at 26℃ in the dark condition. After coculture, cotyledons were rinsed in sterile water containing 500 mg/L carbenicillin and 250 mg/L cefotaxime. In order to induce somatic embryos, the cotyledon explants were cultured abaxial side up on MS selection medium containing 40 mg/L 2,4-D, 100 mg/L kanamycin, added with 500 mg/L carbenicillin and 250 mg/L cefotaxime to prevent *Agrobacterium* growth.

Somatic embryogenesis protocol was followed according to the medthods developed by parrott et al. (1997), and Samoylov et al. (1998). Production of soybean transgenic plant was illustrated in Figure 2.

PCR and Southern blot analysis

Total DNA was isolated from 500 ㎎ of young leaf tissue per sample using the CTAB procedure (Rogers and Bendich, 1988). PCR amplification of γ-TMT transgene fragment in T_0 plants was carried out with the forward (5'-GAA TTC ATG AAA GCA ACT CTA GC-3') and reverse (5'-TAA TCG ATT AGA CTT AGA GTG GCT TC-3') primer pairs. PCR reaction was carried out using PreMix (Bioneer Co., Korea) containing 5 pM primer and 10 ng genomic DNA. Genomic DNA was isolated from young leaf tissues using the CTAB procedure (Rogers and Bendich, 1988). PCR was performed in a thermal cycler for 40 cycles and a cycle was consisted of heat denaturation (94℃, 50 sec), annealing (55℃, 50 sec), and extension (72℃, 1 min) steps. Initial denaturation (95℃, 1 min) or final extension (72℃, 3 min) step was additionally performed in the first and last cycle of the PCR reaction, respectively. For Southern blot, the membrane was hybridized with the *Arabidopsis* γ-TMT gene probe labelled using the AlkPhos Kit (Amersham pharmacia, UK), and the hybrid DNA molecules were detected with CDP-Star detection reagent (Amersham pharmacia, UK).

Analysis of tocopherol

Compositions of tocopherol in the selected transformants were analyzed by high performance liquid chromatography (HPLC). Freeze-dried seeds into fine powder. The dry sample (2.5 g) was weighed into a 25 ml volumetric flask. The sample was brought to volume with 1.5% iso-propyl alcohol in hexane and then vigorously mixed. The mixture was centrifuged at 10,000 rpm and the supernatant was used for tocopherol analysis. The tocopherols were separated and determined according to the method of Carpenter (1979) using HPLC (Hitach Co. Japan). Sample injection was made with a 50 μl injector. The detector was UV detector set at 280 nm (Hitach Co.). Standards for α-, γ- and δ-tocopherol were purchased from Sigma and the standard for each tocopherol was made up in 1.5% iso-propyl alcohol in hexane at concentrations ranging from 50 to 500 ppm.

Results and Discussion

Immature cotyledons of soybean were transformed with *A. tumefaciens* LBA4404 containing the recombinant binary vector pYB-TMT carrying the γ-TMT gene under the CaMV 35S promoter. Santarem et al. (1998) reported that sonication-assisted *Agrobacterium*-mediated transformation (SAAT) method tremendously improved the efficiency of *Agrobacterium* infection by introducing large numbers of microwounds into the target plant tissue. They obtained the best result when immature cotyledons were sonicated for 2 seconds in the presence of *Agrobacterium* (0.11 OD_{600nm}). However, in the preliminary experiments of this study, the best result was obtained with 6 seconds of sonication to the explants. Somatic embryogenesis was conducted as described by Santarem et al. (1998).

After inoculation of *Agrobacterium*, somatic embryogenesis was conducted according to the procedure showed in Fig. 2. Somatic embryos were observed from excised immature cotyledons on MSD40 medium containing 100 mg/L kanamycin. The cotyledon gradually enlarged, swollen and turned brown after 7 days of culture. A few cotyledons turned dark brown after 3 weeks and died. Somatic embryos were observed on the surface of the cotyledon explants after 2-3 weeks of culture and they were light yellow and compact. After 4 weeks of culture, embryos induced on

MSD40 medium were transferred to MSD20 medium containing 20 mg/L 2,4-D, 150 mg/L kanamycin and pH 5.8. They were friable and proliferated on the medium and constituted of clusters of globular somatic embryos with pale yellow. They were subcultured every 2 weeks for 2-3 times. Clumps of embryos were transferred to solid medium (MSM6AC including 200 mg/L kanamycin) or liquid medium (FN Lite or FNL0S3S3GM including 200 mg/L kanamycin) for histodifferentiation and development for 4 weeks. Selections of transgenic embryos were performed in a liquid medium due to higher selection efficiency in solution medium than on solid medium. For maturation, embryos were transferred to maturation medium (MSM6 including 200 mg/L kanamycin) (Finer and Nagasawa 1988).

Somatic embryos were clearly developed from globular stage to cotyledonary stages. The fully matured cotyledon embryos will be ready for desiccation in order to induce roots and shoots of embryo (Parrott et al. 1988; Buchheim et al. 1989). Finally, somatic embryos after desiccation treatment were transferred to germination and regeneration medium (MS0 including 200 mg/L kanamycin) lacking growth regulators in order to induce roots and shoots. The transformed explants were successfully regenerated and were transferred to potting soil after acclimation using a closed hydroponic system in a glass house.

Eleven and eight regenerated plantlets were obtained from Pungsannamulkong and Alchankong, respectively. However, PCR and Southern analyses revealed that only half of the regenerants were positive, giving overall transformation efficiency of 0.7% in the T_0 generation (Table 1). Transgenic soybean plants were successfully transplanted to soil and grown in a glass house. Unfortunately, only a few seeds were set on the T_0 plants (Fig. 3). Hadi et al. (1996) and Trick and Finer (1998) also reported plants regenerated from long-term cultures of soybean were fully sterile and progeny was not recovered. Kanamycin was used as a selection agent in the experiment as kanamycin has been used successfully in recovering stable transformants of soybean (Hinchee et al. 1988; Di et al. 1996). However, Clemente et al. (2000) reported a high frequency of escape plants in kanamycin selection. Therefore, glyphosate selection has been recommended with the *Agrobacterium*-mediated cotyledonary transformation system of soybean. Similarly, Zhang et al. (1999) has used the *bar* gene and the herbicide glufosinate as a selection agent to produce transformed plants with the cotyledonary node system.

Table 1. Frequency of root and shoot formation via somatic embryogenesis from immature cotyledons cocultivated with *Agrobacterium*

Cultivar	Number of cotyledons cultured	Number of embryos		Number of transformed plants (%)
Pungsannamulkong	885	31 (3.5)	11 (1.2)	5 (0.6)
Alchankong	575	15 (2.6)	8 (1.4)	4 (0.7)

Non-transgenic explants could not form roots or though produced roots on the kanamycin selection medium, the roots could not penetrate into solid medim. However, transgenic soybean plant showed very early maturation, and a little decrease in plant height, pod set, ratio of pod set, seed yield and 100-seed weight, compared to normal plants (Fig. 3) (data not shown). Similar results were reported by Hildebrand et al. (1989). They found that the progeny from first generation of regenerants showed greater phenotypic variation than the control population, but the variations for fatty acid and agronomic characters were not observed in the second generation.

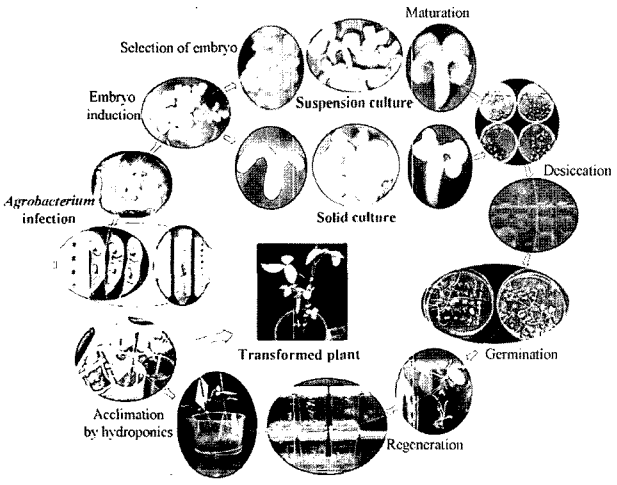

Figure 2. *Agrobacterium*-mediated transformation system via somatic embryogenesis from immature cotyledons of soybean.

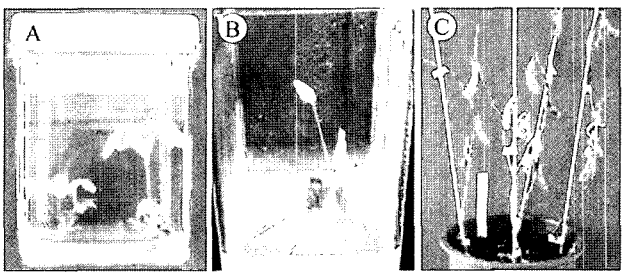

Figure 3. Non-transgenic explant without root (A) and regenerated transgenic soybean plant transformed with *Arabidopsis* γ-TMT cDNA (B, C).

Nineteen regenerated plantlets were obtained on the selection medium from 1,460 cotyledons. PCR was performed to confirm whether the *Arabidopsis* γ-TMT sequence was integrated into the T_0 plants with the genomic DNA isolated from the putative transgenic plants and the primer pairs specifically targeted at the *Arabidopsis* γ-TMT sequence. A fragment in the expected size (1.070 kb) was amplified only from the nine kanamycin-resistant plants but not from the control plants (Fig. 4). It was reconfirmed whether the amplified fragment was from the *Arabidopsis* γ-TMT sequence by Southern blot analysis for the amplified fragment. A hybrid band in the same size as the amplified DNA fragment was detected from 8 out of the 8 PCR-positive plants (Fig. 5). These results indicate stable integration of *Arabidopsis* γ-TMT sequence into soybean genome.

Content of α- and γ-tocopherol isoforms was analyzed as a preliminary test for the expression of the γ-TMT transgene in the 9 putative transgenic lines. Since γ-TMT catalyzes the conversion of γ-tocopherol to α-tocopherol, α-tocopherol content and the ratio of α-/γ-tocopherol can be used as indicators of γ-TMT gene expression. Since each single seed should be saved for the growth of next generation, small samples for analysis were taken from the tip end of the opposite side of radicle. α-Tocopherol content and the ratio of α-/γ-tocopherol in the control plant of Alchankong were 15.2 μg/g and 0.14, respectively. In line AT-1, however, α-tocopherol content was increased about 4.3-fold to 64.8 μg/g. Accordingly, α-/γ-tocopherol ratio also was increased about 8-fold to 1.13 (Table 2; Fig. 6). Overall, α-tocopherol content and the ratio of α-/γ-tocopherol were increased in seeds from plants positive for *Arabidopsis* γ-TMT sequence but with some variations. These results indicate that the γ-TMT transgene is expressed in soybean, but the expression levels may vary among the transgenic lines. Evidences such as transcript levels of γ-TMT transgene and γ-TMT activities will be required to confirm the levels of transgene expression. Recently, Shintani and DellaPenna (1998) reported that overexpression of γ-TMT gene in *Arabidopsis* seed converted γ-tocopherol pool to α-tocopherol. They found that 85-95% of the total tocopherol pool was α-tocopherol in the transgenic seeds, representing an 80-fold increase in α-tocopherol levels compared with the wild-type control. Yun et al. (2001) also reported successful conversion of γ-tocopherol pool to α-tocopherol by the overexpression of Arabidopsis γ-TMT in lettuce and rice.

In conclusion, the results of Southern blot analysis and tocopherol assay suggest the successful integration and expression of γ-TMT transgene in soybean genome.

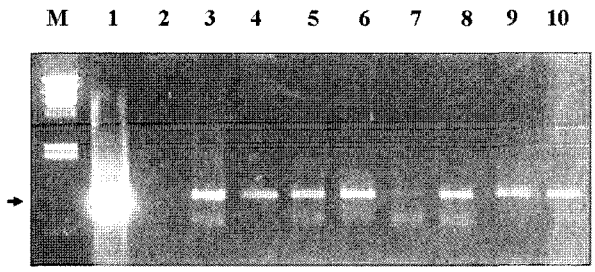

Figure 4. Amplification of a DNA fragment with the specific primer pairs for *Arabidopsis* γ-TMT from the putative transgenic plants via somatic embryogenesis. M, Lamda DNA/*Hind*III ladder; lane 1, amplified product (1,070 bp in size) from the plasmid pBI-TMT containing *Arabidopsis* γ-TMT DNA; lane 2, non-transgenic plant (Alchankong); Lanes 3-6: Transgenic line AT-1, -2, -3, and -4, respectively; Lanes 7-11: Transgenic lines PT-1, -2, -3, and -4, respectively.

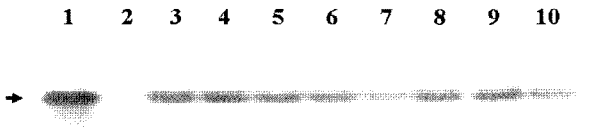

Figure 5. Southern blot analysis from the transgenic T_0 plants with *Arabidopsis* γ-TMT DNA probe. Lane 1, plasmid pBI-TMT containing *Arabidopsis* γ-TMT DNA; Lane 2, non-transgenic plant (Alchankong); Lanes 3 to 11, transgenic plants transformed with pBI-TMT. Lanes 3-6: Transgenic line AT-1, -2, -3, and -4, respectively; Lanes 7-11: Transgenic lines PT-1, -2, -3, and -4, respectively. A band (1.07 kb in size) detected from each line is arrowed.

Table 2. Tocopherol contents and compositions in seeds of the selected transgenic soybean T_0 lines transformed with *Arabidopsis* γ-TMT cDNA

Cultivar	Line	α-Tocopherol (μg/g DW)	α-Tocopherol (μg/g DW)	α-/γ-Tocopherol ratio
Alchankong	wi	15.2	108.3	0.14
	AT-1	64.8	57.3	1.13
	AT-2	51.2	59.4	0.86
	AT-3	13.7	97.4	0.14
	AT-4	41.6	39.1	1.06
Pungsan-namulkong	wi	10.6	111.2	0.10
	PT-1	8.7	122.6	0.07
	PT-2	26.1	58.3	0.45
	PT-3	35.8	49.8	0.72
	PT-4	11.3	95.7	0.12

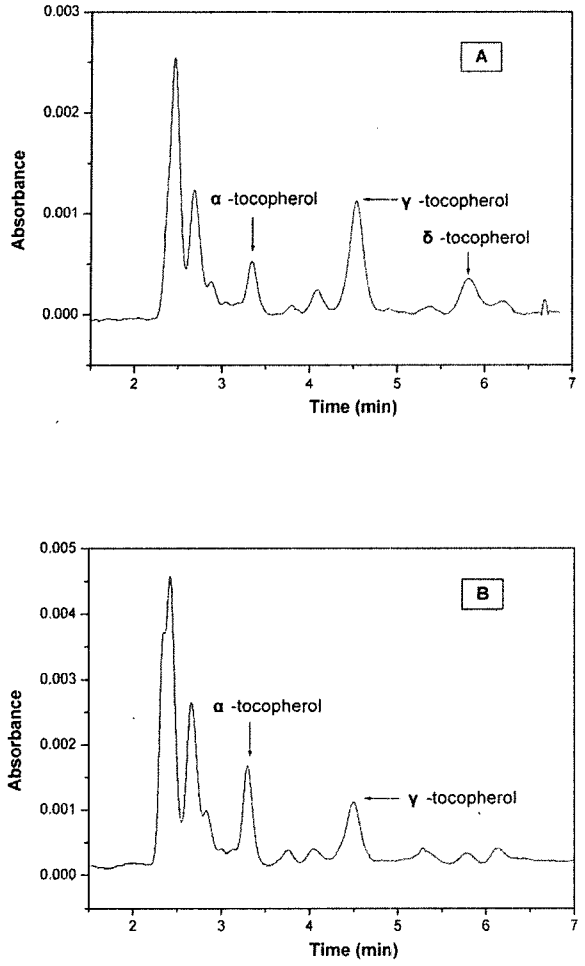

Figure 6. HPLC chromatogram of tocopherol isomers in non-transgenic plant (A) and transgenic line, AT-1 (B). Peak identification: α-tocopherol, RT=3.4; γ-tocopherol, RT=4.5; δ-tocopherol, RT=5.9.

Conclusions

This study was conducted to establish practical molecular procedures for the improvement of soybean seeds. *In vitro* transformation procedures using *Agrobacterium* were established and used to introduce *Arabidopsis* γ-TMT gene into soybean to improve tocopherol composition. Transgenic soybean plants carrying the γ-TMT transgene were obtained using the *Agrobacterium*-mediated transformation system via somatic embryogenesis. Among the transgenic lines, AT-1 and AT-4 had higher levels of α-tocopherol than γ-tocopherol compared to non-transgenic plants and other T_0 lines. Expression of the transgene was at the level to increase the α- to γ-tocopherol ratio about 8-fold from 0.14 to 1.13.

Overall, successful recovery of transgenic lines with improved tocopherol composition from the elite Korean soybean cultivars indicate that the tissue culture and transformation procedures developed in this study are stable and applicable for the transfer of foreign genes into soybean.

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