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Development of Efficient Transformation Protocol for Soybean (*Glycine max* L.) and Characterization of Transgene Expression after *Agrobacterium*-mediated Gene Transfer

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Increasing vitamin E activity in economically important oil crops such as soybean will enhance the nutritional value of these crops. An improved soybean transformation system involving pre-culture of soybean seed on medium supplemented with 1 mg/L 6-benzylaminopurine under dark conditions was established. To improve the nutritional value of soybean by increasing the α-tocopherol content, soybeans were transformed with γ-tocopherol methyltransferase (γ-*TMT*) gene by *Agrobacterium*-mediated transformation. Frequency of soybean transformation was significantly increased from 0.5 to 4.3% by this modified system, and 13 lines of transgenic soybean plants containing γ-*TMT* were obtained. The molecular characterization using polymerase chain reaction (PCR), reverse transcriptase PCR, and Southern blot analysis confirmed insertion and inheritance of the transgene in the transgenic plants and their progeny. Morphologically normal and fertile transgenic plants were analyzed, and the majority of transgenic soybean transmitted herbicide resistance at 3:1 or 15:1 ratios to their progeny. Alpha-tocopherol content in transgenic soybean seeds was determined by high-performance liquid chromatography; over-expression of γ-*TMT* resulted in a 41-fold increase in α-tocopherol over wild-type soybean seeds.

Key words: soybean, tocopherol, transformation

Soybean [*Glycine max* (L.) Merrill] is a legume and an important oil crop worldwide. A typical soybean seed consists of about 40% protein, 20% oil, and 12% carbohydrate on a dry weight basis [Verma and Shoemaker, 1996]; both soybean protein and oil are widely used in food products for human consumption. Vitamin E is a fat-soluble vitamin that acts as antioxidant that protects body tissue from damage caused by unstable substances called free radicals. Soybean oil is also a natural source of vitamin E, which includes a group of structurally related compounds, α-, β-, γ-, and δ-tocopherols. Each form has slightly different biological activity. α-Tocopherol is considered as the most important form of vitamin E for human health, as it has ten-fold higher antioxidant activity than other tocopherols [Traber and Sies, 1996]. γ-Tocopherol has received little attention since the discovery of vitamin E. However, recent studies indicate that γ-tocopherol may also be important to human health and that it possesses unique features that distinguish it from α-tocopherol, such as anti-inflammatory and anticancer activity. However, the bioavailability and bioactivity of γ-tocopherol are lower than those of α-tocopherol [Jiang and Ames, 2003; Jiang *et al*., 2009]. Thus, α-tocopherol is expected to be more potent antioxidant than either β- or γ-tocopherol. The major tocopherol in safflower and sunflower is α-tocopherol (about 80% of total tocopherol content), whereas soybean contains predominantly γ-tocopherol (about 70% of total tocopherol content) [Bramley *et al*., 2000]. Therefore, content of vitamin E in soybean is actually lower than in safflower and sunflower. Thus, many studies on soybean include improvement of nutritional value by increasing α-tocopherol content and enhancing vitamin E activity in seeds.

Development of new varieties of soybean by conventional breeding has been limited by the narrow genetic resource,

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as well as time-consuming and long selection processes. New molecular biology techniques provide additional tools to enhance the efficiency of plant breeding, because they can widen genetic resources and shorten development time. Since the report of the first production of fertile transgenic soybean plant by Hinchee *et al*. [1988], there have been several successful transformations of soybean. James [2009] reported genetically modified (GM) herbicide-tolerant soybean, which has become the major GM crop on 69.2 M ha, or 52% of the world biotechnology crop area. Recent improvements, including modifying the virulence of *Agrobacterium tumefaciens* strains, sonication of explant tissues, and addition of thiol compounds, using embryonic axis, hypocotyls or meristematic tissues of germinating seed have been reported for soybean transformation [Santarém *et al*., 1998; Olhoft *et al*., 2003; Liu *et al*., 2004; Paz *et al*., 2005; Tavva *et al*., 2007; Kim *et al*., 2008; Wang and Yinong, 2008; Jeon EH and Chung YS, 2009]. However, further studies are needed to improve the frequency of soybean transformation.

Molecular biology provides new tools for plant breeding through metabolic pathway engineering of tocopherol biosynthesis and has successfully increased tocopherol content by using the γ-tocopherol methyltransferase (γ-*TMT*) gene to convert γ-tocopherol to α-tocopherol. As a result, γ-*TMT* was cloned from *Arabidopsis thaliana* [Shintani and DellaPenna, 1998], *Perilla frutescens* [Kim *et al*., 2002], enabling the development of transgenic soybean, lettuce, and perilla [Van Eenennaam *et al*., 2003; Lee *et al*., 2007; Lee *et al*., 2008]. The objective of the present study was to increase the frequency of transformation and develop transgenic soybean with high α-tocopherol content by inducing over-expression of γ-*TMT.*

Materials and Methods

Plant material. Mature *G. max* 'Jack' seeds were surface-sterilized with chlorine gas inside a bell jar under a fume hood. Seeds were kept in 100×20 mm Petri dishes with chlorine gas produced by pouring 100 mL of 4% sodium hypochlorite into a beaker and adding 5 mL of 12 N hydrochloric acid. To optimize efficient plant regeneration, soybean seeds were cultured on MS medium supplemented with different concentrations of 6-benzyl-aminopurine (BAP) 0.1-5.0 mg/L under fluorescent light or continuous darkness. The pretreatment condition exhibiting the highest regeneration was considered optimum. Regeneration rate was defined as the percentage of cotyledons that produced fertile plants. Mean number of regenerated plants per cotyledon was obtained by dividing the total number of regenerated plants with the number of regenerating cotyledons in each repetition. After sterilization, seeds were placed on germination medium (GM; MS basal salts with vitamins, 3% sucrose, 0.8% plant agar, and 1 mg/L BAP, optimized from regeneration experiment, pH 5.8) [Murashige and Skoog, 1962]. Seeds were germinated under fluorescent light or darkness at 24±1°C for 5-7 days to compare transformation frequency.

Vector construction. The full-length γ-*TMT* gene obtained from cDNA of *P. frutescens* was inserted at the *Hin*dIII and *Bam*HI sites between the seed-specific vicilin promoter [Higgins *et al*., 1988] and *A. tumefaciens* octopine synthase (ocs) terminator sequences [Barker *et al*., 1983]. The vicilin promoter-γ-*TMT*-terminator cassette was inserted at the *Xba*I site in the multiple cloning site of the pCAMBIA 3301 vector (CAMBIA, Brisbane, Queensland, Australia), which includes an intron containing the *gus* reporter and a phosphinothricin (PPT) selection marker, and the resultant construct was designated as pC3301-TMT (Fig. 1). The construct was transformed into a disarmed *A. tumefaciens* strain EHA105 [Hood *et al*., 1993], which was maintained on a YEP (5 g/L yeast extract, 5 g/L bactopeptone, 5 g/L sucrose, pH 7.2) agar plate containing 50 μg/mL kanamycin and 10 μg/mL rifampicin. A single colony was grown overnight in liquid YEP broth at 28°C with antibiotics, sedimented by centrifugation (3,000×*g*), and resuspended in liquid co-cultivation medium for transformation.

Transformation. The cotyledonary node method described herein was a modified method of Zhang *et al*. [1999]. Two cotyledonary explants were obtained by cutting a horizontal slice through the hypocotyls with a surgical No. 11 blade. The epicotyl was subsequently

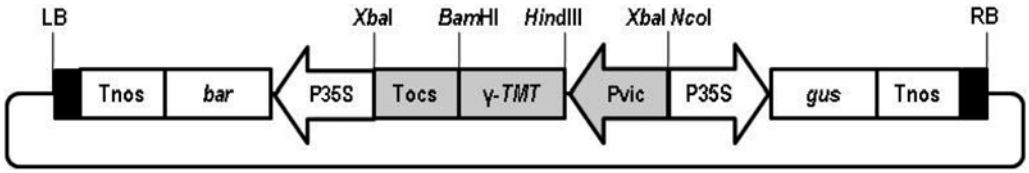

Fig. 1. Schematic representation of the vector constructed for soybean transformation. The T-DNA region of the pC3301-TMT binary vector showing assembly of the *bar* expression cassette, γ-*TMT* expression cassette, and *gus* expression cassette. γ-*TMT*: γ-tocopherol methyltransferase; Pvic: vicilin promoter; Tocs: *Agrobacterium tumefaciens* octopine synthase; P35S: CaMV 35S promoter; *gus*: β-glucuronidase gene; Tnos: *A. tumefaciens* nopaline synthase poly A.

removed, and ten scratches were made at the surface of cotyledonary node regions. Explants were immersed in the *A. tumefaciens* suspension for 30 min, and then ten explants were randomly placed in Petri dishes (100×20 mm) on sterile filter paper placed on solid co-cultivation medium (CM; Gamborg's B5 basal salts with vitamins, 3% sucrose, 20 mM MES, 3.3 mM L-cysteine, 1 mM dithiothreitol, 0.1 mM acetosyringone, 0.8% plant agar, pH 5.4) [Gamborg *et al*., 1968] and incubated at 24±1°C for 5 days under dark conditions.

Selection and plant regeneration. After 5 days of co-cultivation , explants were briefly washed in liquid shoot induction medium (SIM; Gamborg's B5 basal salts with vitamins, 3% sucrose, 3 mM MES, 1.67 mg/L BAP, 250 mg/L cefotaxime, pH 5.7) to remove excess *A. tumefaciens* on explants. Explants were then transferred to solidified SIM without PPT to stimulate shoot induction for the first 14 days, after which the explants were sub-cultured on fresh SIM containing 5 mg/L PPT for selection of transformed shoots. Organogenic shoots from the explants were trimmed and then transferred to shoot elongation medium (SEM; MS basal salts with vitamins, 3% sucrose, 3 mM MES, 0.5 mg/L giberellic acid, 50 mg/L asparagine, 1 mg/L zeatin, 0.1 mg/L indole-3-acetic acid, 250 mg/L cefotaxime, 50 mg/L vancomycin, 0.8% plant agar, 5 mg/L PPT, pH 5.7). Explants were transferred to new SEM medium every 14 days, and surviving shoots were planted on root induction medium (RIM; MS basal salts with vitamins, 3% sucrose, 1 mg/L naphthalene acetic acid, 0.8% plant agar, pH 5.7) and grown until roots developed. After acclimation, the transgenic plants were transplanted to potting soil and maintained in a greenhouse. beta-glucuronidase (GUS) activity in tissues was determined via a histochemical assay according to the method of Jefferson *et al*. [1987]. The leaves of transformed or wild-type soybean plants were immersed in freshly prepared X-gluc (5-bromo-4-chloro-3-indolyl-β-glucuronide) solution and incubated overnight at 37°C.

PCR and Southern blot analysis. Genomic DNA was extracted from 0.5 g soybean leaves using the cetyl-trimethylammonium bromide (CTAB) method [Rogers and Bendich, 1988]. Polymerase chain reaction (PCR) was performed with 100 ng of genomic DNA as template using primers specific to *bar* and *gus*. The primers employed were bar-F, 5'-TCTCGGTGACGGGCAGG-3'; bar-R, 5'-CTCGTCCGTCTGCGGGA-3'; gus-F, 5'-GTG CACGACCACGCATTAATGGACT-3'; gus-R, 5'-AAAT CGCCGCTTTGGACATACCATC-3'. PCR was performed in a thermal cycler for 30 cycles, and each cycle consisted of heat denaturation (94°C, 30 s), annealing (58°C, 30 s), and extension (72°C, 30 s) steps. Initial denaturation (95°C, 5 min) and final extension (72°C, 10 min) steps were additionally performed in the first and last cycle of the PCR reaction, respectively. For Southern blot, 20 μg of genomic DNAs from transgenic soybeans (T_0 and T_1) were digested with the restriction enzyme *Nco*I or *Xba*I, and the digested DNA was separated on a 0.7% (w/v) agarose gel and blotted onto a Hybond N^+ nylon membrane (Amersham Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. The probe γ-*TMT* gene was labeled using the RediPrimeII random prime labeling system (Amersham Pharmacia), and the hybrid DNA molecules were detected with Molecular Imager FX (BioRad, Hercules, CA).

RT-PCR analysis of gene expression. Total RNA was isolated from T_1 and wild-type soybean seeds by using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. RNA was pretreated with RNase-free DNase (Qiagen) to remove any residual genomic DNA. cDNA obtained from this RNA using Accupower RT-PCR kit (Bioneer, Daejon, Korea) was then used as template for PCR reaction using the primer set (TMT-F, 5-ATGATTGAGGAGTCCCTCCG-3 and TMT-R, 5-TATAACTGCCGGCCAAAATG-3), with *lectin* as a control. PCR conditions used were 94°C for 5 min for initial melting, followed by 30 cycles of amplification with each cycle consisting of the following steps: 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 10 min.

Analysis of tocopherol. Tocopherols were analyzed by the modified method of Kamal-Eldin *et al*. [2000]. Approximately 0.5 g of T_1 was ground in liquid nitrogen, and the resultant powder was mixed with 5 mL hexane in a 15-mL test tube by vortexing and incubated for 3 h at room temperature. The hexane extract was filtered through a Sep-Pak NH_2 cartridge and dried at 60°C for 30 min. After drying, each sample was diluted in 2 mL hexane, and 20 μL of each diluted sample was directly injected for high-performance liquid chromatography (HPLC) analysis. The standard α-, β-, γ- and δ-tocopherols were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). The HPLC analysis was performed on a Shimadzu system (Kyoto, Japan) equipped with a SPD-7AV UV detector, LC-7A pump, SCL-6B system controller, and C-R6A integrator. The column used was Supelcosil LC-NH_2 (250×4.6 mm, 5 μm; Bellefonte, PA) operated at 30°C. A mixture of hexane:ethyl acetate (70:30, v/v) was used as mobile phase at a flow rate of 1 mL/min, and detection was accomplished by monitoring at 292 nm.

Results

To optimize conditions for soybean transformation, the effects of parameters that influence transformation frequency

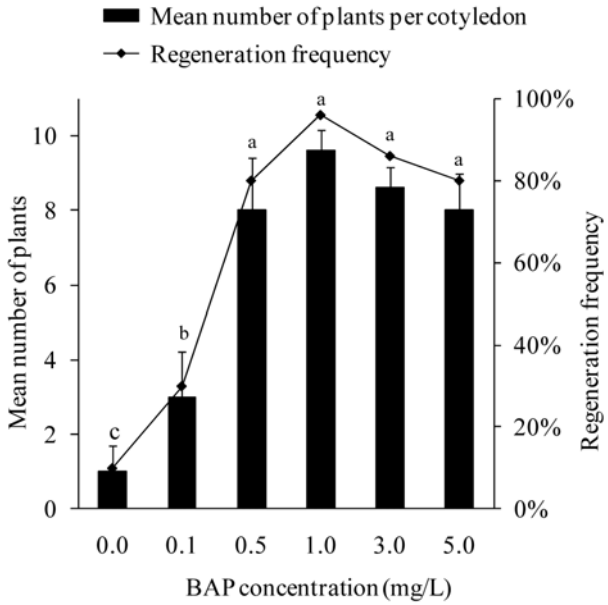

Fig. 2. Effect of plant regeneration from pre-cultured soybean seed on germination medium supplemented with 0-5 mg/L BAP followed by culture on regeneration medium. Data represent the averages of five replicates. Bars topped with the different letters show significant differences among treatments according to LSD at α=0.05.

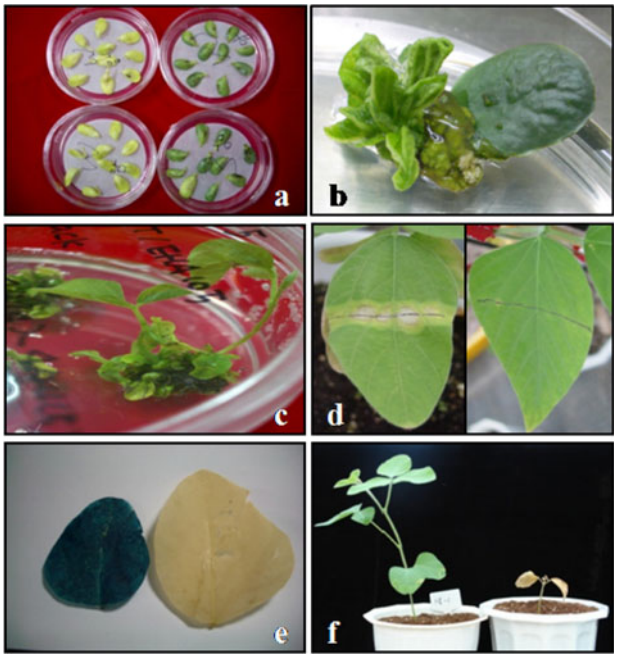

Fig. 3. Transformation of soybean using a modified method. Explants were excised from pre-cultured soybean seed on GM supplemented with 1 mg/L BAP in the dark, inoculated with *A. tumefaciens*, and co-cultivated in CM (a). Explants were embedded in SIM to stimulate *de novo* shoot formation (b). Explants were cultured on SEM for shoot elongation 3 months after co-cultivation (c). 0.3% BASTA was applied to a leaf at the seed maturity stage (d). Transgenic soybean was stained for GUS expression (e). A transgenic soybean plant with BASTA resistance (left) and wild-type plant (right) (f).

were examined. Soybean seeds were germinated on medium with different concentrations of BAP to determine the effect of BAP on the cotyledonary node regeneration during germination. The number of regenerated shoots depended on BAP concentration, such that, at 0.1-3.0 mg/L BAP the number of regenerated shoots increased dramatically, and then decreased at 5.0 mg/L. The highest regeneration frequency was observed at 1 mg/L BAP (Fig. 2). The results showed that BAP at 0.1-5.0 mg/L significantly increased regeneration frequency compared to the control. In preliminary experiments, we established that dark treatment during germination also affects soybean transformation.

To investigate the effects of BAP and dark treatment during germination on soybean transformation, four independent experiments were performed (Table 1). Final transformation frequency based on the number of BASTA-resistant transgenic plants per number of infected explants obtained from different experimental conditions ranged from 0.5 to 4.3%. The highest transformation frequency was observed with condition 4, in which soybean was germinated on medium supplemented with 1 mg/L BAP in dark conditions. The dark treatment during germination increased transformation frequency (2.7, 4.3%), whereas the continuous fluorescent light led to a frequency of only 0.5, 1.1%, in agreement with the results of Barwale *et al.* [1986], who found that exposure of soybean explants to a dark period for initial induction of shoot formation was necessary. Addition of 1 mg/L BAP to the germination medium also increased transformation frequency from 0.5 to 1.1% (under light condition) or 4.3% (under dark condition). The combination of dark treatment with 1 mg/L BAP had a synergistic effect and increased transformation frequency, as was also found in other legumes [Lippmann and Lippmann, 1984; Malik and Saxena, 1992; Saini *et al*., 2003]. Based on statistically significant results obtained for soybean transformation conditions, dark treatment with BAP is more effective in transformation than without BAP. No statistical differences were found in the continuous light treatment; nevertheless, a positive effect of BAP on transformation frequency was noted.

To increase α-tocopherol content in soybean seed using *Agrobacterium*-mediated transformation, *P. frutescens* γ-*TMT* cDNA was cloned at the *Hin*dIII-*Bam*HI site in the pCAMBIA 3301 vector between the seed-specific vicilin promoter and ocs terminator sequences. The construct, pC3301-TMT, was transformed into a disarmed *A. tumefaciens* strain EHA105, which was subsequently

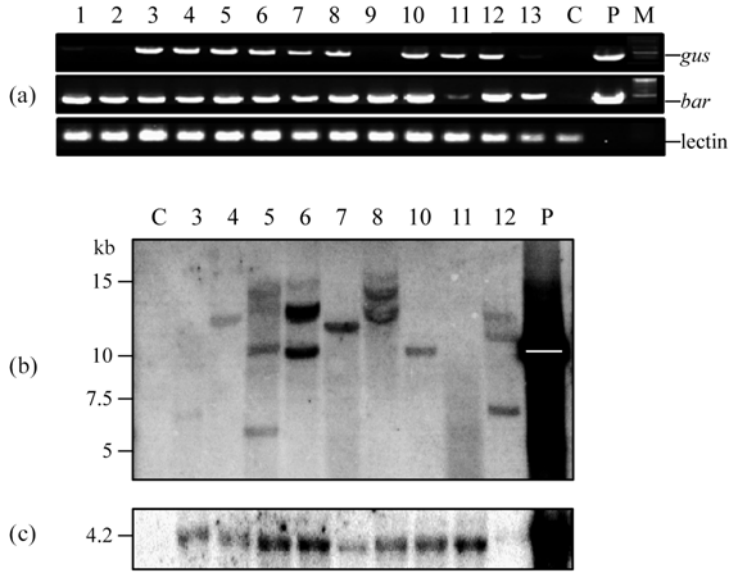

Fig. 4. PCR amplification and Southern blot analyses of transgenic soybean plants transformed with the pC3301-TMT. (a) Representative PCR analysis of genomic DNA to detect the presence of *gus* and *bar* genes in putative transgenic plants. PCR amplification of the 500-bp fragment of the *gus* gene, 400-bp fragment of the *bar* gene and 118-bp fragment of the endogenous soybean lectin gene. (b) Southern blot analysis of *Nco*I restricted total genomic DNA isolated from putative transgenic soybean plants (T_0) and hybridized with a γ-*TMT* cassette probe. (c) Southern blot analysis of *Xba*I restricted genomic DNA isolated from nine different T_1 progeny and hybridized with the γ-*TMT* gene probe. Lanes 1-13 represent DNA samples isolated from putative transgenic soybean plants; Lane C: wild-type soybean; Lane P: pC3301-TMT; Lane M: molecular marker.

used to transform the cotyledonary node of soybean. Using this optimized protocol, transgenic soybean plants showing BASTA resistance were obtained from cotyledoanry node explants (Fig. 3a). The shoots emerged after 4-5 days, could be regenerated directly from whole cotyledonary nodes without going through a callus stage (Fig. 3b). Most shoots elongated on SEM to 3-4 cm in length within 6 weeks (Fig. 3c). Healthy leaves of transgenic and wild-type plants were selected for leaf painting and were brushed with 0.3% BASTA. All transgenic soybean plants showed resistance to BASTA; wild-type soybean showed necrosis (Fig. 3d), and transgenic progenies (T_1) were BASTA-resistant (Fig. 3f). To determine transmission of the transgene, the T_1 generation was analyzed for BASTA resistance (Table 2); the analysis confirmed resistance and verified the functional expression of the selectable marker, *bar*, in the transgenic soybean plants. The soybean transformed with pC3301-TMT was confirmed using GUS staining of leaf tissue. Transgenic soybean leaves exhibited GUS expression that was clearly distinguishable from that of the wild-type plant (Fig. 3e).

The soybeans transformed with *A. tumefaciens* EHA105 harboring pC3301-TMT were confirmed by PCR amplification and genomic Southern blot analysis. Thirteen putative transgenic plants derived from BASTA-resistant plants were selected and checked by PCR. All 13 plants showed the expected 400-bp fragment for *bar* and 118-bp fragment for the endogenous soybean *lectin*; however, two plants (lanes 2 and 9) did not contain the 500-bp fragment for *gus* (Fig. 4a). These PCR results confirmed that 11 of the 13 regenerated plants, approximately 85%, contained the full transgene derived from the pC3301-TMT vector. Nine randomly sampled *gus* plants, PCR-positive for *bar*, were further tested using Southern blot analysis. Genomic DNAs of transgenic soybean (T_0) or wild-type plants were digested at the *Nco*I site in the pC3301-TMT vector and hybridized with the radiolabeled γ-*TMT* probe. One to four copies of the γ-*TMT* gene were integrated into the genome of transgenic soybean plants, whereas no hybridization signal was detected in wild-type soybean (Fig. 4b). The number of hybridization bands reflected the number of insertion loci of the transgenes in the plant genome. The frequency of single insert was about 33%, and the other plants had more than two transgene loci. The results of Southern blot analysis confirmed the presence and integration of the transgenes in the transgenic soybean plants. Furthermore, Southern blot analysis of nine transgenic soybean (T_1) progeny that were positive for BASTA resistance using *Xba*I as a restriction enzyme showed that all inherited the γ-*TMT* gene (Fig. 4c). More than one copy of the inserts was found in T_0 plants when restricted with *Nco*I (Fig. 4b), whereas restriction with *Xba*I resulted in a single copy. Southern blot showed the presence of the transgene in

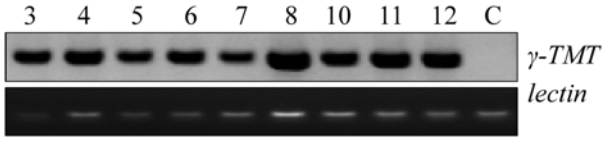

Fig. 5. RT-PCR analysis for the expression of γ-*TMT* gene (upper panel) of different transgenic lines and expression of *lectin* as a control (lower panel).

nine T_1 plants derived from transgenic soybean (T_0), also confirming stable inheritance of the transgene in the progeny generation. In order to confirm the stable expression of γ-*TMT*, transgenic soybean seeds were subjected to RT-PCR analysis (Fig. 5). A gene-specific band was observed in all nine transgenic soybeans, but was absent in the wild-type soybean. Results showed a correlation between the expression levels and the copy number; expression level increased with increasing number of copies.

The concentration and content of tocopherol isolated from transgenic soybean seeds (T_1) are presented in Table 3. α-Tocopherol content increased dramatically in transgenic soybean seeds (40.9-77.2%) relative to controls (1.5%). In control seed, tocopherol content was 15.9±1.4 ng/mg α-tocopherol and 667.0±1.5 ng/mg γ-tocopherol; the most abundant tocopherol in wild-type soybean seeds was γ-tocopherol. In seeds derived from transgenic soybean plants, a significant increase in α-tocopherol due to conversion from the γ- to the α-form was observed: 318±1.7-656.0±6.7 ng/mg α-tocopherol and 5.8±1.8-228.0±15.2 ng/mg γ-tocopherol.

Discussion

Despite the efforts of many researchers, progress in developing transgenic soybean plants has been hampered by the very low transformation frequency. On the other hand, commercialized transgenic soybeans have been cultivated since 1996 [Padgette *et al*., 1995]. Transgenic soybeans have been obtained using *Agrobacterium*-mediated DNA delivery methods, bombardment of shoot meristems [McCabe *et al*., 1988], and biolistic-mediated methods [Finer and McMullen, 1991]. The most widely used system for soybean transformation is based on *A. tumefaciens*-mediated transferred DNA (T-DNA) genes into cell axillary meristems of the cotyledonary node [Hinchee *et al*., 1988], although success has been limited to a few cultivars. Recently, a number of successful transformation protocols have been developed, but much improvement is needed for application to the soybean transformation. In the present study, establishment of a highly efficient and repetitive regeneration system in soybean without manipulation of plant growth regulators such as thidiazuron (TDZ) [Franklin *et al*., 2004; Shan *et al*., 2005] and carbohydrates in the medium [Sairam *et al*., 2003] is described. The transformation frequency was increased by pretreatment of the seeds on germination medium supplemented with 1 mg/L BAP and dark treatment, comparable to the procedure developed by Zeng *et al*. [2003] (Table 1), ranging from 1.1 to 4.3%, depending on the treatment tested. Plant growth regulators, mainly cytokinins, in the regeneration medium play an important role in shoot induction, development, and multiplication for transformation from cotyledonary node explants [Lippmann and Lippmann, 1984; Barwale *et al*., 1986; Malik and Saxena, 1992]. Pre-culture of explants on medium supplemented with BAP prior to bacterial inoculation has been shown to increase genetic transformation frequency in many plants [Birch, 1997], because it stimulates cell division and shoot formation, and thus competence for transformation.

Generally, germination proceeds under fluorescent light in culture rooms, but pre-culture of seed in darkness was critical to improve soybean transformation frequency in the present study. Maturity of tissue is strongly related to cell competence for transformation, and higher T-DNA deliveries are found in yellow tissues than in green tissues. This indicates the need to expose soybean seed to darkness, as suggested by Barwale *et al*. [1986]. Our results confirmed that a combination of a dark treatment and 1 mg/L BAP was effective in increasing the frequency of soybean transformation. Moreover, the combination of BAP with pre-culture of soybean in darkness had a

Table 1. Effect of light and BAP on soybean transformation and frequency of soybean transformation

Experiment	Treatment		No. of explants infected	No. BASTA resistant	Transformation frequency (%)[a]
	Light	BAP			
1	+	-	204	1	0.5
2	+	+	187	2	1.1
3	-	-	149	4	2.7*
4	-	+	141	6	4.3**
Total			681	13	1.9

[a]Data were analyzed using procedure for LSD. Significance at 5% and 1% indicated by * and **, respectively.

Table 2. BASTA resistance segregation analysis of T_1 progeny of five transgenic soybean plants

T_0 plant	T_1 segregation No. (BASTA resistance +: -)	Expected ratio	Chi-square	*P*-value
3	35:3	15:1	0.18	0.98
4	18:2	3:1	2.4	0.12
6	19:1	15:1	0.05	0.99
7	25:12	3:1	1.09	0.30
10	30:7	3:1	0.73	0.39

Table 3. Summary of HPLC data on tocopherol content in seeds collected from transgenic soybean plants

Plant No.	α-tocopherol (ng/mg seed)	β-tocopherol (ng/mg seed)	γ-tocopherol (ng/mg seed)	δ-tocopherol (ng/mg seed)	α-tocopherol (%)	γ-tocopherol (%)
3	600±2.2	169±4.8	8±1.5	-	77.2	1.0
4	372±1.3	187±2.1	18±11.3	-	64.5	3.1
6	656±6.7	208±21.7	5.8±1.8	14±2.9	74.2	0.7
7	462±21.3	186±20.0	19±1.2	-	69.2	2.8
10	318±1.7	167±16.5	228±15.2	65±2.7	40.9	29.3
Control	15.9±1.4	27±1.9	667±1.5	330±6.3	1.5	64.1

Data represent the average of three replicates±SD

synergistic effect, further increasing the transformation frequency.

All 13 plants that exhibited BASTA resistance also showed amplification of the *bar* gene in PCR analysis, thus confirming the presence and expression of the transgene in transgenic soybean plants. Genomic DNA derived from independent transgenic soybean plants should yield unique patterns when digested with *Nco*I, and the number of bands should reveal the approximate copy number. The number of hybridization signals indicates that three plants had a single copy, and the others had two or more copies of T-DNA integrated in their genomes. Southern blot analysis of nine transgenic soybean plants revealed different patterns of junction fragments between the T-DNA and the plant genome, depending on the integration site (Fig. 4b). These results also indicate that transgenic soybean plants were derived from independent transformation events. The sizes of the bands detected were larger than those of the γ-*TMT* probe (4.2 Kb), thereby confirming integration of T-DNA into the plant genome.

Among the four tocopherols found in plants, α-tocopherol is the predominant form in leaves, whereas γ-tocopherol is the most abundant form in seeds [Shintani and DellaPenna, 1998; Grusak and DellaPenna, 1999]. To generate transgenic soybean plants with increased α-tocopherol, over-expression of the γ-*TMT* gene in seeds was induced using a seed-specific promoter. The present study showed that seed-specific over-expression of the *P. frutescens* γ-*TMT* gene increased the α-tocopherol content from 15.9±1.4 ng/mg in wild-type seed to 656.0±6.7 ng/mg in transgenic soybean seed (Table 3). In transgenic soybean seed, α-tocopherol content was 74.2% of that of the total tocopherol, which corresponds to 41-fold increase in α-tocopherol compared to wild-type soybean seed. In addition, γ-tocopherol, which was 64.1% of the tocopherol content of wild-type seed, decreased to 0.7% of total tocopherol in the transgenic soybean seed. Alpha-tocopherol displays the highest vitamin E activity, whereas β-, γ-, and δ-tocopherol activities are 50, 10, and 3%, respectively, of the vitamin E activity of α-tocopherol [Kamal-Eldin and Appelqvist, 1996]. Given the differing vitamin E potencies of α-, β-, γ-, and δ-tocopherol, targeted over-expression of the γ-*TMT* gene was calculated to have increased vitamin E activity of transgenic soybean seed by 7.2-fold compared to vitamin E biological activity of wild-type seed. Recently, Tavva *et al*. [2007] reported that transgenic soybean with increased α-tocopherol content (450 pmol mg^{-1}) was developed by expressing a perilla γ-*TMT* gene using direct DNA delivery. In the present study, however, *Agrobacterium*-mediated transformation resulted in high α-tocopherol content (656.0±6.7 ng/mg), which corresponds to a 3.3-fold increase in α-tocopherol content compared to that of Tavva *et al*. [2007], which showed the transfer of relatively low copy number than with direct DNA delivery. The mechanism and biological activity for this difference is not clear; thus, additional experiments need to be done to confirm these results.

In summary, an improved soybean transformation system involving pre-culture of soybean on medium with 1 mg/L BAP under dark condition was established. The

applicability of this system was proved through the increased α-tocopherol content of the transgenic soybean. This transgenic soybean could be used to improve nutritional value for human health and as a germplasm resource for soybean breeding. Furthermore, this system could provide a useful tool for both improvement of cultivar quality and functional genome research in soybean.

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