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Field Performance and Morphological Characterization of Transgenic *Codonopsis lanceolata* Expressing γ-TMT Gene.

Bimal Kumar Ghimire*[1], Cheng Hao Li, Hyun Young Kil*, Na Young Kim***, Jung Dae Lim*, Jae Kwang Kim****, Myong Jo Kim*, Ill Min Chung*****[1], Sun-Joo Lee*****, Seok Hyun Eom*, Dong Ha Cho*, and Chang Yeon Yu*†**

*Bioherb Research Institute, Kangwon National University, Chunchon 200-701,Korea.
**Key Laboratory of Forest Genetics and Tree Breeding, Northeast Forestry University, Harbin 150040, China.
***Department of Food & Nutrition, Kyunghee University, Seoul 130-701, Korea.
****National Institute of Agricultural Biotechnology, RDA, Suwon 441-707, Korea.
*****Department of Applied Life Science, Konkuk University, Seoul 143-701, Korea.

ABSTRACT : Field performance and morphological characterization was conducted on seven transgenic lines of *Codonopsis lanceolata* expressing γ-TMT gene. The shoots were obtained from leaf explants after co-cultivation with *Agrobacterium tumefaciens* strain LBA 4404 harboring a binary vector pYBI 121 that carried genes encoding γ-Tocopherol methyltransferase gene (γ-TMT) and a neomycin phosphotransferase II gene (npt II) for kanamycin resistance. The transgenic plants were transferred to a green house for acclimation. Integration of T-DNA into the T_0 and T_1 generation of transgenic *Codonopsis lanceolata* genome was confirmed by the polymerase chain reaction and southern blot analysis. The progenies of transgenic plants showed phenotypic differences within the different lines and with relative to control plants. When grown in field, the transgenic plants in general exhibited increased fertility, significant improvement in the shoot weight, root weight, shoot height and rachis length with relation to the control plants. However, all seven independently derived transgenic lines produced normal flower with respect to its shape, size, color and seeds number at its maturity. Indicating that the addition of a selectable marker gene in the plant genome does not effect on seed germination and agronomic performance of transgenic *Codonopsis lanceolata.* T_1 progenies of these plants were obtained and evaluated together with control plant in a field experiment. Overall, the agronomic performance of T_1 progenies of transgenic *Codonopsis lanceolata* showed superior to that of the seed derived non-transgenic plant. In this study, we report on the morphological variation and agronomic performance of transgenic *Codonopsis lanceolata* developed by *Agrobacterium* transformation.

Key words : Transgenic plants, field evaluation, morphological characteristics, γ-TMT

INTRODUCTION

Codonopisis lanceolata (Companulaceae) is a perennial herb, distributed widely in East Asia (Hong *et al.*, 1984). The roots of *C. lanceolata* have been used as a remedy for dyspepsia, poor appetite, fatigue and psychoneurosis (Zhang 1982) and as a herbal drug to treat bronchitis, cough, spasm, and inflammation (Lee *et al.*, 1985, Lee *et al.*, 2007). Genetic transformation is one of the most attractive method for plant breeding since it introduce agronomically important trait to pre-existing genotypes within a short period as compared to traditional breeding methods (Suzuki *et al.*, 2002). However, retaining desirable traits in the germplasm while introducing novel genes is a major consideration for all transgenic crop improvement programmes (Manickavasagam *et al.*, 2004). *Agrobacterium tumefaciens* mediated transformation with important agronomic trait have been reported for several plant species and transformed plants exhibiting alternation in morphological characters and agronomic performance such as, in rice (Qing-yau Shu *et al.*, 2002), in tobacco (Alekseeva *et al.*, 2004), in broccoli (Chen *et al.*, 2004). An altered phenotype due to the expression of γ-TMT gene have been reported for several important species, such as in *Perilla frutescens* (Tarra *et al.*, 2007), in lettuce (Cho *et al.*, 2004), in *Brassica juncea* (Yusuf *et al.*, 2006). There have been few reports on the regeneration of transgenic plants in *Codonopsis lanceolata* (Min *et al.*, (1992), Shin *et al.*, (2000), Cho *et al.*, (1999), but till to date, there are no reports on evaluation of morphological characters. Moreover, it is necessary to investigate the suitability of biotechnology approach to the plant and to determined the performance of greenhouse transgenic plant as well as its environmental risk and plant pest problem in the field. In the present study, morphological characters were compared, its possible causes analysed in transgenic and non-transgenic

[1]These two authors are equally contributed in this study.
† Corresponding author: (Phone) +82-33-255-6411 (E-mail) cyyu@kangwon.ac.kr
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control plant, and also examined whether an alteration of tocopherol composition by γ-TMT gene influences morphological characteristics and agronomical performance of the transgenic plants.

MATERIALS AND METHODS

Agrobacterium tumefaciens strain and plasmid vector

Agrobacterium tumefaciens strains LBA 4404 harboring the binary vector pYBI121 was used which contains the neomycin phosphotransferase gene (*npt* II) directed by the nos promoter as selectable marker. A single colony of *Agrobacterium tumefaciens* strains LBA 4404 was grown for 24 hrs at 28 ± 1 ℃ with shaking (150 rpm) in 20 ㎖ of liquid Luria- Bertani (LB) medium (10 g/ℓ tryptone, 10 g/ℓ NaCl, 5 g/ℓ yeast extract, pH 7) containing 100 ㎎/ℓ kanamycin. The *Agrobacterium tumefaciens* cells were spun down by centrifugation for 10 min at 7,000 × g at 4℃ and resuspended in liquid inoculation medium (MS salt with 20 g/ℓ of sucrose) to obtain a final $O.D_{600}$ of 1.0 for plant infection.

Transformation procedure

To test the importance of preculture, leaf explants of 3-4 weeks old *in vitro* grown *Codonopsis lanceolata* were injured slightly across the midrib with a sterile scalpel and transferred into pre-culture medium containing 0.1 ㎎/ℓ NAA and 1 ㎎/ℓ BAP, 3% sucrose and 0.8% agar at pH 5.8 for various period (i.e. 0, 24, 48 and 72 h) prior to inoculation in bacterial culture. To investigate the influence of inoculation time, the leaf explants which had been pre-cultured for various period were then soaked in the bacterial culture with different growth phase (OD_{600} values of 0.5, 0.6, 0.8, 1.0 and 1.2) for 1, 2, 5, 8 and 10 minutes. The infected explants were dry-blotted by using sterile filter paper and transferred to co-cultivation medium containing 0.1 ㎎/ℓ NAA and 1 ㎎/ℓ BAP, 3% sucrose and 0.8% agar at pH 5.8. To investigate the influence of co-cultivation period, leaf explants were inoculated for 5 min, were then transferred to co-cultivation medium for 1, 2, 3, 4 and 5 days in the dark at 25℃.

After co-cultivation, the explants were washed in liquid MS medium supplemented with filter sterilized 250 ㎎/ℓ cefatoxime, blotted dry with sterile filter paper and transferred to selection medium, consisting of MS salt containing 0.1 ㎎/ℓ NAA and 1 ㎎/ℓ BAP, 3% sucrose and 0.8% agar at pH 5.8, supplemented with 100 ㎎/ℓ kanamycin for selection and 250 ㎎/ℓ cefotaxime to eliminate bacterial growth. The culture were kept under 16 h photoperiod under 45 μmol m^{-2} s^{-1} at 25 ± 2 ℃ and they were transferred onto fresh selection medium with 50 ㎎/ℓ kanamycin without cefatoxime and allowed to regenerate for 30 days with sub-culturing after every two weeks. The primary regenerated shoot from the leaf explants was then transferred in the MS and 1/2 MS media supplemented with 50 ㎎/ℓ kanamycin for root induction. The rotted plantlets were than transferred to the pots 85 ㎝ diameter plastic pot containing sterilized soil mixture (vermiculite and perlite 1 : 1) with polyethylene bags to maintain high humidity and kept at 25 ± 2 ℃ in a growth chamber for one week and then transferred to the green house.

DNA isolation and polymerase chain reaction

In order to verify the presence of the *npt* II gene and γ-TMT gene in a regenerated plant, total DNA was extracted from young leafs excised from kanamycin resistant shoots as well as from control plant by CTAB (cetyl Trimethyl Ammonium bromide) method (Murray and Thompson (1980). Two primers used for PCR amplification of the 700 bp of the *npt* II gene were designed as: N-1 (5′-GAA-GCT-ATT-CGG-CGG-CTA-TGA-CTG-3′) as a sense primer and N-2 (5′-ATC-GGG-AGC-GGC-GGC-GAT-ACC-CTA-3′) as a anti-sense primer. Amplification conditions were 35 cycle each consisting 1 min, at 94℃, 1 min at 60℃ and 1 min 30 s at 72℃ and with a final extension at 72 ℃ for 10 min. The primers for a 1,070 bp fragments of the

TMT-1 primer (5-GAA-TTC-ATG-AAA-GCA-ACT-CTA-GC-3) as a sense primer and TMT-2 primer (5-TAA-TCG-ATT-AGA-CTT-AGA-GTG-GCT-TC-3) as a anti-sense primer. Amplification condition for γ-TMT gene fragment were 35 cycle each consisting 50 s at 94℃, 50 s at 57℃, 1min at 72℃, post elongation 72 ℃ for 3 min and with a final extension at 72 ℃ for 10 min. Amplification products were analyzed on 1% (w/v) TAE agarose gels.

Southern blot analysis

Southern blot analysis was carried out with the genomic DNA extracted from transgenic and untransformed (control) plants according to the method of Sambrook and Russell (2001). Total genomic DNA from the leaf tissue of putatively transformed and untransformed control plants was extracted by CTAB method and digested by *Eco RI* and *Xba I*. The digested DNA was separated by electrophoresis on 0.8% (w/v) agarose and transformed to a positively charged nylon membranes (Hybond-NX, Amersham Biosciences, UK). The membrane was incubated for 3 hrs at 65 C in prehybridization solution (5 × SSC, 0.1% N-Lauroyl Sarcosine, 0.02% SDS and 1% blocking solution). The probe used to identify the γ-TMT gene was obtained from digestion of the plasmid vector (pYB1130) by *Eco RI* and *Xba I*. The probe was labeled according to the manufacturer's instructions using a non-radioactive DIG DNA labeling and Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). After prehybridization, the denatured probe was added to the hybridization solution for 16 hrs at 65 ℃. After hybridization, the membrane was washed twice in 5 X SSC, 0.1% SDS for 5 min at room temperature, then twice with a mixture of 0.1 × SSC and 0.1% SDS for 15 min at 60 ℃, and subjected to detection

of probe using a non-radioactive, DIG DNA labeling and Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Morphological and agronomic characteristics

In order to evaluate morphological and agronomic performance of transgenic *Codonopsis lanceolata*, eight plants of each PCR positive T1 transgenic lines and seed derived control plants were transferred to pot (20 ㎝ × 19 ㎝) containing sterilized bed soil (vermiculite and perlite 3 : 1). Plants were grown in a shade house and evaluated for morphological characters including shoot height, shoot weight, number of leaf, leaf length, leaf width, length of internodes, flower size, flower shape, anther number, stigma number, root weight root diameter, number of lateral root and weight of 1,000 seeds. Seed harvested from individual T_0 transgenic plants were sown in a seedling bed and transplanted after 4 weeks to the field in early May. Weeds around the field were controlled by herbicides and in the pot were removed by hand. Plant height of the different genotypes was measured from the base of stem to the tips of longest leaf at its maturity. Root and shoot mass was obtained after drying the plants at 40 ℃ for at least 24 h.

Statistical analysis

All experiments were repeated at least three times. The data shown represent the mean ± S.E. The data were statistically analyzed using one-way ANOVA.

RESULTS

PCR analysis

DNA isolated from transformed plants, non-transformed plants, and plasmid pYBI121 was used as template DNA for PCR amplification of γ-TMT gene. The presence of a band of 1070 bp in samples from transformed shoots (lane 4-11) confirmed the integration of γ-TMT gene. Whereas, amplification of γ-TMT gene was not observed in non-transformed control plant (Fig. 1)

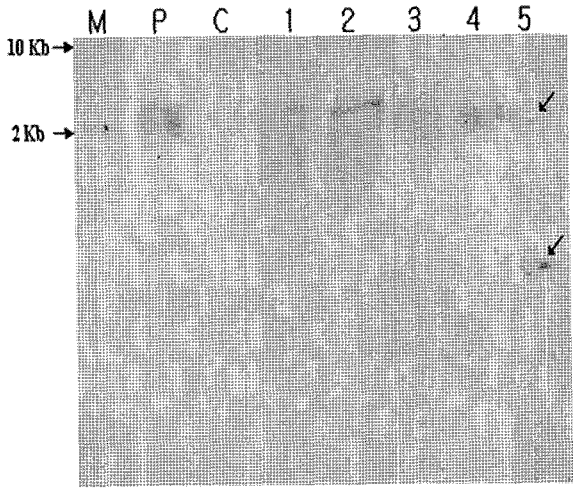

Fig. 2. Southern hybridization analysis of DNA isolated from leaves of putative transformed *Codonopsis lanceolata* plants. Up to 20 ㎍ per lane of genomic DNA was digested with *Eco RI and Xba I*, fractionated with 0.8% agarose gel and blotted onto a nylon membrane. M: molecular weight markers; P: plasmid DNA (pYBI121) was digested with *Eco RI and XbaI* and used as positive control; C: DNA from untrans-formed leaf tissue as negative control; Lane 1-5: genomic DNA of putative transgenic lines 2, 3-1, 5-1, 16-1 and 16-2 plants respectively. Fig. 3. Comparative morphology of leaf from T_1 generation of *Codonopsis lanceolata* under field test. (a) Leaf from control untransformed plant, (b-h) Leaf from transgenic *Codonopsis lanceolata* line 3-1, 3-2, 4, 5-1, 11-1, 16-1, 16-2 respectively.

Morphological and Agronomic Analysis of Transgenic *Codonopsis lanceolata*

Transformed plantlets were acclimatized into a greenhouse prior to use them into field trail. In order to evaluate morphological characteristics of transgenic *Codonopsis lanceolata*, eight plants of each transgenic lines and untransformed control plants were transferred to field. Plants were grown in a shade house and evaluated for different morphological characteristics including shoot height, shoot weight, leaf length, leaf number, leaf width, root length, root weight, root diameter, weight of 1000 seeds, number of lateral root, shape and size

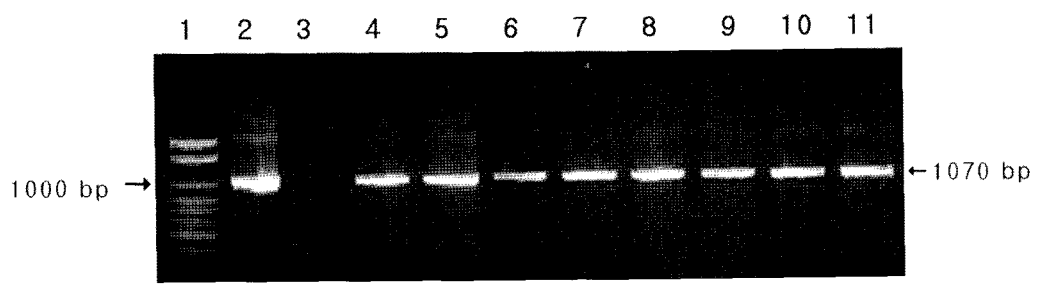

Fig. 1. PCR analysis from leaves of transgenic *Codonopsis lanceolata* using specific primers of amplification of the 1070 bp fragment of γ-TMT gene. Lane 1, Molecular marker; lane 2, the plasmid pYBI121 as a positive control; lane 3, the genomic DNA from untransformed plants (Negative control); lane 4-11, the genomic DNA of T_0 transformants.

of flower, number of stigma and anther. Phenotypic differences were observed within the different transgenic lines and between the transgenic and untransformed control plants. The overall performance of most transgenic lines was superior to the non-transformed controls in terms of number of leaf, root weight. Similarly, significant differences in shoot height and root length were observed between transgenic lines and control plant, where most transgenic lines showed inferior characters over control plant. Whereas, there was no apparent differences in terms of flower shape, color of flower, anther number and stigma number per flower between transgenic and control plants (data not shown). This is consistent with the observation that transgenic plants showed more leaf width, root diameter and number of lateral root than untransformed control plant but the differences was not significant (Table 1). Nearly all of the seven transgenic lines showed a reduced shoot weight compared to their control, with the exception of transgenic line 4, 5-1 and 16-2.

Variation in leaf color in transgenic lines observed either yellowish (line 4 & 5-1) or dark green in line 11-1 transgenic plant (Fig. 3). Similarly, differences in 1000 seed weight were observed in the field trials of transgenic lines and control plants (Fig. 4).

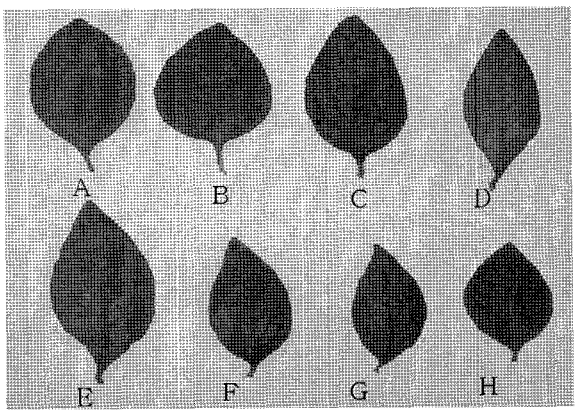

Fig. 3. Comparative morphology of leaf from T1 generation of *Codonopsis lanceolata* under field test. (a) Leaf from control untransformend plant, (b-h) Leaf from transgenic *Codonopsis lanceolata* line 3-1, 3-2, 4, 5-1, 11-1, 16-1, 16-2 respectively.

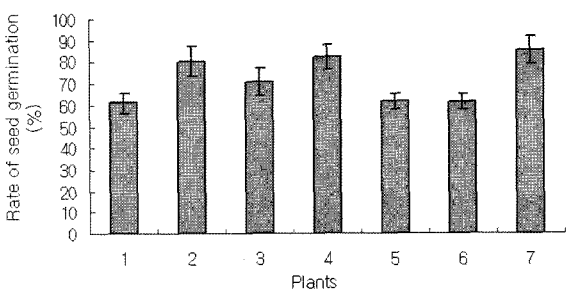

Fig. 4. Rate of T_1 seed germination of *Codonopsis lanceoalata*. (1) Control untransformed plants. (2-7) represents the line 3-1, 4, 5-1, 11-1, 16-1, 16-2 of transgenic *Codonopsis lanceolata* respectively.

Most of the transgenic lines showed increased in 1000 seed weight except in 16-2 transgenic line. Transgenic seed appeared dark brown in color with similar in size and shape with that of control plant.

The evaluation of T1 transgenic seed germination in the field showed no differences among most of the transgenic lines and showed normal growth habit with respect to untransformed control seed germination but transgenic line 11-1 and 16-1 showed about a 61% reduction in germination rate than the rest of the transgenic line (Fig. 4). All transgenic lines flowered and matured at around same time as untransformed control plant. All transgenic lines set seed. There were differences in fertility and alteration in flower size of some lines (Fig. 5-G). Mean height of mature transgenic lines ranged from 43.0 cm (line 16-1) to 133.33 cm (line 4) was due to longer internodes of its genotypes. On the basis of root fresh weight, only one transgenic line (3-2) showed lowest mean weight (1.98 g) and reduced root length & diameter among all the transgenic line tested in the field (Table 1 & 2).

Flower morphology of transgenic lines was similar to that of control plants except in genotype 3-1 and 16-2, where size of the flower was smaller than that of the control plants (Fig. 5-G). Similarly, the roots regenerated from transgenic lines were highly branched with increased number of lateral roots (Fig. 6.).

DISCUSSION

Evaluation of transgenic plants under field conditions is necessary to determine the effect of transgene on the agronomic traits of crop, so as to verify that such plants will not pose environmental risks (Barro *et al.*, 2002, Wang *et al.*, 2003). An altered phenotype due to the expression of γ-TMT gene has been reported for several important species, such as in *Perilla frutescens* (Tarra *et al.*, 2007); in lettuce (Cho *et al.*, 2004); in *Brassica juncea* (Yusuf *et al.*, 2006). In the present report, we evaluated morphological and agronomic characteristics of seven independent line of transgenic *Codonopsis lanceolata* with that of untransformed control plant in the field. The result obtained from the field test showed variation in the important agronomic traits but well within acceptable agronomic standard. None of the transgenic plants showed weediness.

It is well known that prolonged undifferentiated stage (callus phase) increases the soma clonal variation (Larkin and Scowcroft 1981) because the somaclonal variation happened throughout the culture process (Koeppler *et al.*, 2000) The tissue culture condition, particularly callus induction, represents a physiological stress that is characterized by dissruption of normal developmental control. Moreover, the occurrence of somaclonal variation is influenced by genotypes and tissue

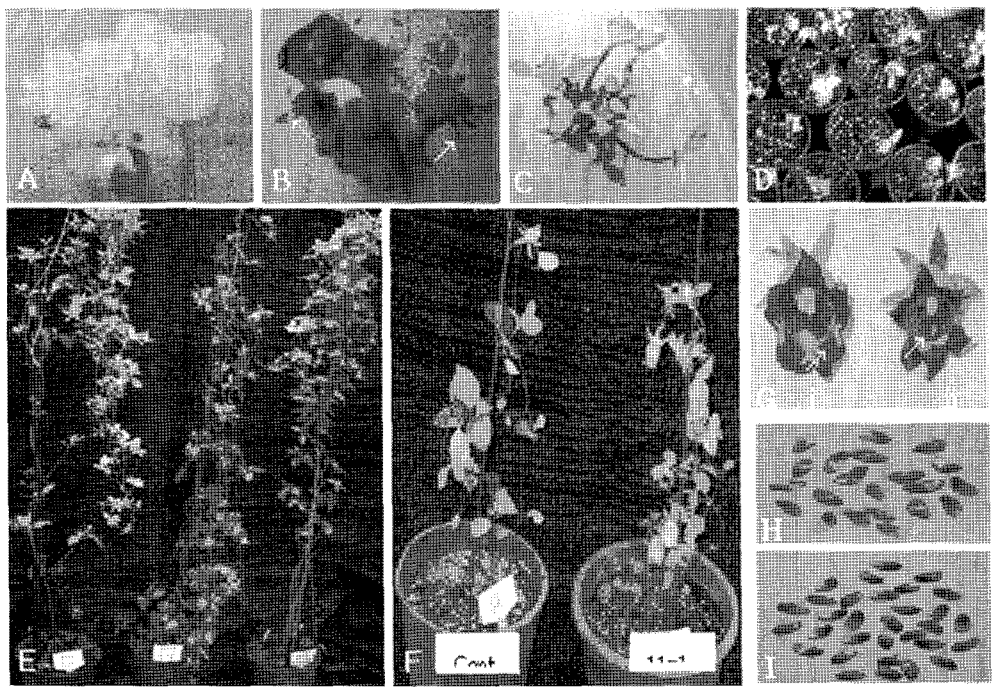

Fig. 5. Regeneration and field evaluation of transgenic *Codonopsis lanceolata*. (A) Embryogenic callus developed from the leaf explants, (B) Regeneration of putative transgenic shoots from the embryogenic callus of leaf explants, (C) Clusters of putative transgenic shoots on selection medium, (D) Transgenic plants growing in the greenhouse, (E) Growth of primary transgenic *Codonopsis lanceolata* in a shade house, (F) Growth of T_1 transgenic *Codonopsis lanceolata* in a shade house, (G) A flower of a control plant (i) and a transgenic plant (ii). Arrows indicate the stamens. (H) Seeds of control untransformed plant, (I) Seeds of transgenic *Codonopsis lanceolata*.

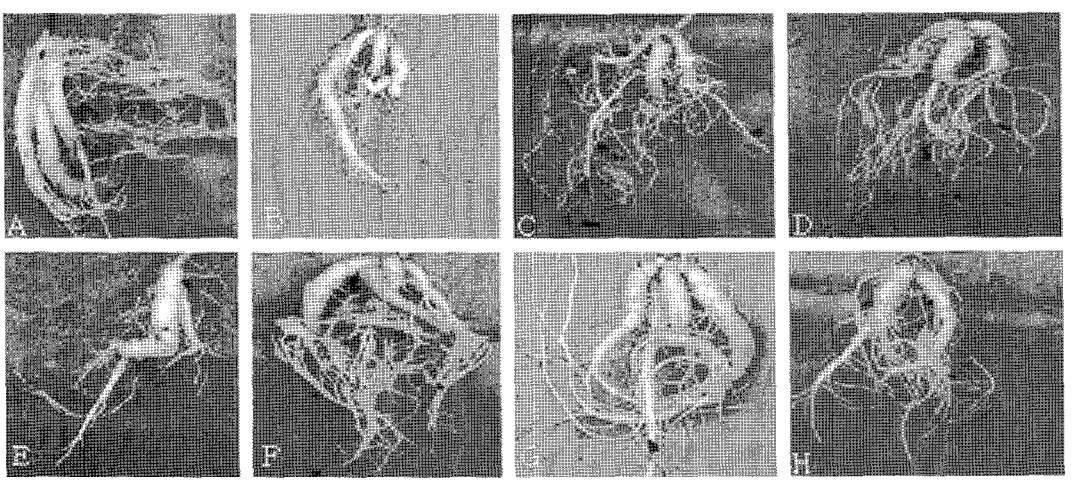

Fig. 6. Comparative morphology of root from T_1 generation of *Codonopsis lanceolata* under field test. (a) Root from control untransformed plant, (b-h) Root from transgenic *Codonopsis lanceolata* line 3-1, 3-2, 4, 5-1, 11-1, 16-1, 16-2 respectively.

sources (Karp, 1995). Yet to be investigate in *Codonopsis lanceolata*, the co-culture of calli and *Agrobacterium tumefaciens* may also induce mutation as reported by Dale and Mc Partlan (1992) in potatoes. Also, the variation might have occurred due to the break down of plant genes caused by transgene insertion (van Liasebetens *et al.*, 1993) or due to transgene induced endogene silencing (Matzke *et al.*, 2000).

In this work, the high frequency of variation might be due to longer undifferentiated phase (callus stage) and prolonged subculturing of regenerants during *Agrobacterium* transformation process that could increase the somaclonal variation in different tansgenic line. Guo *et al.*, (2006) reported apparent genomic variation in the 63 randomly tagged regenerants of *Codonopsis lanceolata* when compared with the single mother donor plant by two molecular markers.

In this report, the transgenic plants had more dry mass and leaf length than control plants. Increased photosynthetic rate per leaf area or an increase in total leaf area can yield

Table 1. Morphological variations of untransformed and transgenic *Codonopsis lanceolata* expressing γ-TMT gene.

Line	Height of Shoot (cm)	Number of Leaf	Length of Leaf (cm)	Weight of Shoot (g)	Length of Internode (cm)	Width of Leaf (cm)	Length of Rachis (cm)
Control	113.33 ± 7.21	51.00 ± 9.10	7.27 ± 0.42	11.67 ± 3.84	8.50 ± 0.43	4.03 ± 0.26	8.73 ± 0.51
3-1	73.33 ± 5.94	38.00 ± 3.31	6.40 ± 0.08	10.33 ± 3.53	6.50 ± 0.20	4.03 ± 0.09	7.60 ± 0.88
3-2	54.67 ± 6.69	44.00 ± 8.65	7.07 ± 0.19	6.19 ± 2.26	12.17 ± 1.25	4.37 ± 0.07	9.67 ± 1.19
4	133.33 ± 8.31	98.00 ± 5.10	7.73 ± 0.24	19.77 ± 4.68	10.10 ± 0.48	3.93 ± 0.21	11.27 ± 0.12
5-1	65.00 ± 4.72	64.00 ± 7.73	8.03 ± 0.24	17.81 ± 4.22	8.33 ± 0.95	4.40 ± 0.08	11.50 ± 0.41
11-1	116.83 ± 3.31	97.00 ± 8.27	6.73 ± 0.30	8.02 ± 1.53	11.17 ± 0.36	3.63 ± 0.15	4.93 ± 1.25
16-1	43.00 ± 6.95	62.67 ± 7.85	5.70 ± 0.20	8.42 ± 2.39	7.90 ± 0.55	0.26 ± 0.15	8.67 ± 0.19
16-2	84.67 ± 3.60	56.00 ± 4.55	6.27 ± 0.31	11.90 ± 2.9	7.80 ± 1.31	0.36 ± 0.21	8.67 ± 1.21

[a] Data were recorded 6 months after transfer to field test. Values represent the mean ± standard error of at least 8 plants for each control or transformed plant line.

Table 2. Morphological variations of untransformed and transgenic *Codonopsis lanceolata* expressing γ-TMT gene.

Line[a]	Size of Flower	Weight of Root (cm)	Diameter of Root (cm)	Length of Root (cm)	Number of lateral Root	Weight of 1000 Seeds (g)
Control	Normal	4.25 ± 0.79	1.03 ± 0.29	10.83 ± 0.49	1.33 ± 0.27	2.24
3-1	Small	3.82 ± 0.29	0.87 ± 0.09	9.50 ± 1.43	2.00 ± 0.47	2.80
3-2	Normal	1.98 ± 0.07	0.70 ± 0.13	5.97 ± 0.87	2.00 ± 0.82	2.87
4	Normal	7.02 ± 0.69	1.43 ± 0.21	4.63 ± 0.59	2.00 ± 1.25	2.94
5-1	Normal	5.23 ± 0.49	0.90 ± 0.09	11.67 ± 0.72	1.67 ± 0.27	2.88
11-1	Normal	3.89 ± 1.29	0.80 ± 0.13	7.33 ± 0.54	2.33 ± 0.72	3.04
16-1	Normal	5.22 ± 0.54	0.83 ± 0.19	8.10 ± 0.77	1.67 ± 0.27	ND
16-2	Small	5.13 ± 0.45	0.93 ± 0.07	7.40 ± 0.57	2.67 ± 0.27	1.96

ND-Not determined

[a] Data were recorded 6 months after transfer to field test. Values represent the mean ± standard error of at least 8 plants for each control or transformed plant line.

increased dry weight, however, an increase in the photosynthetic rate requires increase enzymatic function as well as changes in the leaf structure to allow adequate rate of CO_2 diffusion (Nobel, 1977; Bjorkman, 1981) There were no significant differences in the root diameter, number of lateral root, leaf width, flower shape and size between the transformed and control plants. Whereas, in terms of shoot height, shoot weight, root weight, and root length, a significant phenotypic alterations among the transgenic lines were observed as compared to the control plants. Similar results have previously been reported for a number of plant species in *Hyoscyamus muticus*, (Sevon *et al.*, 1997) in *Angelonia salicriifolia* (Koike *et al.*, 2003) in *Festuca arundinacea* (Wang *et al.*, 2003), in rice (Shu *et al.*, 2002). It has previously been demonstrated that expression of γ-TMT gene in the plants causes no specific difference in the important agronomic traits, such as in *Perilla frutescens* (Venkata *et al.*, 2007), in Brassica (Yusuf *et al.*, 2006), indicating that the variation in the various transgenics was independent from transgene insertion. In the field trials, the transgenic lines had an increased 1000 seeds weight; except in genotype 11-1 and 16-1. Variation in the seed weight among the different transformants was also been reported in transgenic Golden Promise (Bregitzer *et al.*, 1998).

In summary, T_1 progenies of transgenic *Codonopsis lanceolata* showed variation in agronomic performance to that of the seed derived non-transgenic plant. However, most of these differences were not significant. Indicating that, routine insertion of novel transgene does not affect agronomic performance of the plant.

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