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AGROBACTERIUM TUMEFACIENS MEDIATED TRANSFORMATION OF CHICORY (*CICHORIUM INTYBUS* L.) WITH *MYTILUS GALLOPROVINCIALIS* FOOT PROTEIN TYPE FIVE (MGFP-5)

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Abstract

Mytilus galloprovincialis foot protein type 5 (Mgfp-5) can be used as medical adhesive or in underwater environments due to its significant adhesive ability and biodegradable properties. To obtain sufficient Mgfp-5 for further study, genetic engineering has been abundantly used, while plant genetic engineering was rarely applied. This study reports an efficient protocol for the direct regeneration of chicory (*Cichorium intybus* L.), with a regeneration frequency of 79.18% in the case of inoculation on MS+1.5 mg/L 6-BA+0.2 mg/L NAA medium. The plant expression vector pRI101-Mgfp of the *Mgfp*-5 gene was transformed into chicory via *Agrobacterium* mediation. After plant selection and regeneration, regenerated plants with resistance were successfully obtained. The efficient transformation system of the *Agrobacterium*-mediated genetic transformation consists of leaf explants immersed in *Agrobacterium* at $O.D._{(600)}$ of 0.6 for 10 min after being pre-cultured for 3 d, followed by co-culture for 3 d. Then, these explants were transferred to medium containing 50 mg/L kanamycin[1] selection power. As proved via PCR, RT-PCR, and Western blot analysis, the *Mgfp*-5 gene was integrated into the chicory genome, accompanied by normal transcript and expression, as well as stable genetic transformation and inheritance. These results underpin future research toward providing a feasible method for the production of Mgfp-5 protein.

Key words: *Mytilus galloprovincincialis* foot protein type five (Mgfp-5), *Agrobacterium tumefaciens*, Genetic transformation, *Cichorium intybus* L.

Abbreviations: MS, Murashige and Skoog medium; CSM, Callus and Shoot multiplication medium; 6-BA, 6-Benzylaminopurine.; NAA, Naphthalene Acetic Acid; CTAB, Cetyltrimethyl ammonium bromide; $O.D._{(600)}$, Optical Density at a wavelength of 600 nm; RT-PCR, Reverse Transcription Polymerase Chain Reaction; YEB, Yeast-Extract Broth; Cef, Cefotaxime; Rif, Rifampicin; Kan, Kanamycin; Str, Streptomy.

Introduction

Mussels are widely distributed shellfish in marine environments. The mussel foot produces and secretes byssus, with the main component of *Mytilus* adhesive protein (MAP) or *Mytilus* foot protein (Mfp). MAP is able to form not only permanent and strong, but also flexible underwater bonds to a variety of substrates, such as glass, plastic, metal, and teflon. Moreover, their biodegradable properties include a strong antioxidant capacity, no toxicity, and no immunogenicity. Therefore, MAP can be used as medical adhesive, as underwater coating, and underwater adhesive (Waite & Tanzer, 1981, Nicklisch *et al*., 2013, Hwang *et al*., 2004, Hwang *et al*., 2007(a), Hwang *et al*., 2007(b), Miller *et al*., 2015(a), Miller *et al*., 2015(b)). The contents of MAP mainly include six types of adhesion proteins (MAP-1, 2, 3, 4, 5, and 6) and three types of precollagen (preCoI-D, preCoI-P, and preCoI-NG) (Lucas *et al*., 2002). Among them, MAP-5 is considered as the main molecule adhesion protein for the highest content of 3, 4-dihydroxyphenyl-L-alanine DOPA (30%) (Liao *et al*., 2007).

Previous studies on MAP mainly focused on extraction, separation, and the mechanism of adhesion in animals (Choi *et al*., 2012, Li *et al*., 2011, Lu *et al*., 2013). Researchers have also successfully used genetic engineering to produce mussel adhesion proteins. Several MAPs such as Mgfp-5, fp-151, Mgfp-3A, and fp-353, were produced through a prokaryotic expression system (Hwang *et al*., 2004, Hwang *et al*., 2005, Hwang *et al*., 2007(a), Gim *et al*., 2008). Moreover, recombinant Mcfp-3 was successfully expressed in yeast (Platko *et al*., 2008). However, there are some shortcomings for the expression of the recombinant protein in *Escherichia coli* and yeast. For example, modification and fold of the recombinant protein may be erroneous in *E. coli* Secreted proteins and therefore can be easily hydrolyzed by enzymes in yeast. To overcome these disadvantages, the chicory (*Cichorium intybus* L.) plant has been chosen to express the recombinant protein. Molecular farming in plants has already proven to be a successful method for producing a range of technical proteins and the aspired proteins could be produced safely, inexpensively, and in almost unlimited quantity using only simple nutrients, water, and sunlight. (Larrick *et al*., 1998, Vermij & Waltz, 2006, Golovkin *et al*., 2007, Ma *et al*., 2003, Fischer & Emans, 2000). Our previous work has successfully expressed *Mytilus galloprovincincialis* foot protein type (Mgfp-5) in tobacco and obtained Mgfp-5 transgenic tobacco (Lv *et al*., 2016).

Chicory is a herbaceous plant and an important vegetable crop cultivated throughout Asia and Europe (Bais & Ravishankar, 2001). Chicory contains a large number of pharmaceutically important compounds and its crude protein content is 6.44-27.35% (Zhang *et al*., 2015). Most importantly, it serves as a reactor plant for plant genetic engineering due to its high transformation efficiency and strong genetic ability (Rehman *et al*., 2003, Buhara *et al*., 2007, Matvieieva *et al*., 2011, Matvieieva *et al*., 2015). Here, chicory leaf explants were selected as receptor and *Agrobacterium* mediation was used for the expression of the protein Mgfp-5. This is the first time that chicory was used for the synthesis of transgenic plants with the *Mgfp*-5 gene, mediated via *Agrobacterium*.

Materials and Methods

Plant materials: Chicory seeds were washed with 75% ethanol for 30 s, followed by surface sterilization using 0.1% (w/v) $HgCl_2$ for 7 min. After thoroughly rinsing five times with sterile water, the treated seeds were placed on Murashige and Skoog medium (Murashige & Skoog, 1962), supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar, with the pH adjusted to 5.8 ± 0.2 (MS). The seeds were allowed to grow into full plantlets.

Leaf explants (0.5 cm × 0.5 cm) excised from growing plants was transferred onto MS supplemented with 6-benzylaminopurine (6-BA) and Naphthalene Acetic Acid (NAA) (callus and shoot multiplication medium, CSM). The leaves were regenerated on the medium for callus and shoot induction. Moreover, adventitious buds with an approximate length of 1.5-2 cm were excised and transferred to 1/2 MS containing NAA in the range of 0.1-0.4 mg/L for root induction. All cultures were maintained under cool fluorescent light (3000 lux) with 16L: 8D-h photoperiods at 25 ± 2°C. Well-developed root plantlets were transferred into small pots for establishment in a green house.

Optimization of kanamycin (Kan) concentration for selection of transformants: The leaf explants were placed on CSM containing different concentrations of Kan (5-70 mg/L) to determine the optimum dose for the selection of putative transformants. The results were recorded after 5-6 weeks of culture.

Agrobacterium mediated transformation: The plasmid pRI101-Mgfp contained Mgfp-5 gene and six His-Tag under the control of the CaMV 35S promoter and the NOS terminator. The plasmid was introduced into the LBA4404 *Agrobacterium tumefaciens* strain using the freeze-thaw method (Lv *et al*., 2016). In the LBA4404 suspension, chicory was transformed through the leaf-disc infection technique (Horsch *et al*., 1985). Leaf discs were pre-cultured in CSM for time periods ranging from 1 to 4 days, after which, they were immersed in a bacterial suspension and were surface-dried on sterilized filter paper.

To determine the optimum bacterial concentration and infection time, experiments were conducted using different *Agrobacterium* suspensions, $O.D._{(600)}$ (0.4, 0.6, and 0.8). The leaf explants were exposed to bacterial inoculums with $O.D._{(600)}$ 0.6 for different durations of 5, 10, and 15 min. Leaf segments and bacteria were co-cultured for 1 to 4 days at 25°C in the dark on MS without antibiotics.

The treated leaf segments were transferred onto the optimum CSM fortified with 500 mg/L cefotaxime (Cef) and 50 mg/L Kan for direct regeneration. Surviving shoots were transferred into the rooting medium (MS containing 50 mg/L Kan and 0.1 mg/L NAA) for root induction when they had elongated to 1.5-2 cm in length. Well-rooted plantlets were transferred into pots, grown in the greenhouse and T_1 generation seeds were obtained via hand-pollination. T_1 individuals were surface sterilized and sowed on MS containing 50 mg/L Kan.

Generation of transgenic plants and genetic analyses: The total genomic DNA was extracted from fresh leaves of transformed and non-transformed chicory plants using the CTAB protocol (Khan *et al*., 2007). To confirm the presence of the *Mgfp*-5 gene, polymerase chain reaction (PCR) was used with the following primerset designed for the *Mgfp*-5 gene: The forward primer was 5'-GGAATTCCATATGAG TTCTGAAGAA-3' and the reverse primer was 5'-GGGGTACCCTAATGGTGATGGTG-3'. Plant total RNA was isolated using the Trizol protocol (Wu *et al*., 2012). Genomic DNA was removed and then, 5 µg RNA was used as template for reverse transcription (RT) according to the Primer Script™ II Strand cDNA Synthesis Kit protocol (Takara Corp, China). Then, the cDNA was used as template for RT-PCR reactions. Furthermore, the PCR products were examined via electrophoresis on a 1% agarose gel.

Progeny segregation analysis for Mgfp-5 gene expression: Seeds from T_0 plants were germinated in MS supplemented with 50 mg/L Kan, while seedlings from untransformed plants were used as negative control. The segregation ratio of the trans-gene was recorded after 60 days (Saha *et al*., 2014). Surviving seedlings were further PCR analyzed for *Mgfp*-5 gene.

Total plant proteins were extracted from leaves of T_1 generation chicory according to Festa *et al*., (2013). Leaves were ground in liquid nitrogen under addition of Polyvinylpolypyrrolidone cross-linked (PVPP) and were then immersed in extraction buffer. The buffer/leave ration was 600 µL / 0.6 g. The buffer was composed of 20 mmol/L Tris-HCl pH 8.0, 0.7 mol/L sucrose, 5% w/v SDS, 1 mmol/L Ethylene Diamine Tetraacetic Acid (EDTA), 1 mmol/L phenylmethylsulphonyl fluoride (PMSF), and 4% v/v β-mercapthoethanol. The homogenates were centrifuged at 11,000 g for 30 min at 4°C and the supernatants were precipitated with cold (-20°C) acetone. Dried pellets were separated via SDS-PAGE (15% separation gel and 5% stacking gel) and Western blot. For Western blot analysis, proteins were transferred onto a nitrocellulose membrane (Immobilon - P) using a Mini-Trans blot cell (Bio-Rad) and transfer buffer (2.5 mol Tris, 19.2 mol glycine, 20% methanol) for 1 h at100 V. The proteins of interest were detected using anti His-Tag Mouse Monoclonal Antibody (1/3000 v/v, Tianjin Sungene Biotech Co., Ltd.) and Peroxidase-Conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (1/5000 v/v, ZSGB-BIO). The membrane was then washed and the protein was visualized via ECL.

Results

Optimization of chicory regeneration: Chicory seed germination required 4-7 days (Fig. 1A). The leaf explants of aseptic seedlings were cultured on MS supplemented with various concentrations of 6-BA and NAA for callus induction (Fig. 1B). Among all treatments (Table 1), green calluses were generated at the leaf explants after 7 d of callus induction and differentiation detected under different concentrations of plant growth regulators. The changes of callus

induction rate did not significantly increase with the concentration of 6-BA and NAA. Calluses increased with increasing 6-BA concentration under the same NAA concentration. The lowest induction rate was 86.33%. There was a maximum callus induction rate at 98.00% on MS with 2.5 mg/L 6-BA and 0.2 mg/L NAA (Fig. 1C). However, after about 14 d, most calluses ceased growing. They turned brown and finally died (Fig. 1D). Only 19.63% callus of A7 could initiate shoot regeneration, while the percentage of shoot induction of A3 was 79.18%. Therefore, the optimal medium was MS containing 1.5 mg/L 6-BA and 0.2 mg/L NAA for shoot regeneration (Fig. 1E).

The regenerated shoots were cut cultivated on rooting medium, which was 1/2 MS with different concentrations of NAA (Table 2, Fig. 1F). Low NAA concentration promoted rooting, while high NAA concentration inhibited root growth. Within 30 days of culture in the medium with 0.1 mg/L NAA, the rooting rate of regeneration shoots reached 97.33% (Table 2, Fig. 1G). Moreover, well-developed root plantlets were planted into pots, followed by cultivation in the greenhouse (Fig. 1H, I).

Optimization of Kanamycin concentration for selection of putative transformants: The leaf explants were highly sensitive to increasing Kan concentrations. A preliminary study was performed to test increasing concentrations of Kan: 5, 10, 20, 30, 40, 50, 60, and 70 mg/L (Fig. 2). At 5, 10, and 20 mg/L, most of the leaf explants were green and a high regeneration frequency of green calluses was achieved (Fig. 1J). However, most of the leaf discs did not form calluses at levels above 50 mg/L (Fig. 1M). Toxicity effects in the callus tissues were observed as early as during the incubation period, and proceeded rapidly. At all concentrations, the physical appearance of the calluses cultured on Kan-containing media was significantly different from that of calluses cultured on control medium contained shortening, bleaching, yellowing, and browning (Fig. 1J, K, L, and M). Increasing Kan concentration was associated with a gradual decrease in the number of calluses per segment, clearly indicating the toxicity of Kan (Fig. 2). 50 mg/L Kan was found sufficient to kill untransformed calluses and was therefore chosen as the optimum concentration for the selection of transformed plants (Fig. 1N, O, and P).

Table 1. Effect of MS medium fortified with different concentrations of 6-BA and NAA on callus and shoots regeneration from the leaf explants of chicory.

No.	6-BA (mg/L)	NAA (mg/L)	Number of leaves	Percentage of callus induction (%)	Percentage of shoots induction (%)
A1	1.0	0.2	50	86.33 ± 5.23^{a}	39.72 ± 6.79^{bcd}
A2	1.0	0.5	50	89.67 ± 6.74^{a}	47.37 ± 6.80^{de}
A3	1.5	0.2	50	95.67 ± 2.84^{a}	79.18 ± 5.58^{f}
A4	1.5	0.5	50	96.33 ± 1.15^{a}	62.05 ± 5.65^{ef}
A5	2.0	0.2	50	96.67 ± 2.40^{a}	42.75 ± 7.20^{abc}
A6	2.0	0.5	50	97.67 ± 1.85^{a}	26.04 ± 6.70^{abc}
A7	2.5	0.2	50	98.00 ± 1.00^{a}	19.63 ± 5.45^{a}
A8	2.5	0.5	50	95.67 ± 2.85^{a}	22.89 ± 3.20^{ab}

Percentage of callus induction (%) = the number of callus/the number of leaf explants×100%
Percentage of shoots induction (%) =the number of shoots of callus/the number of callus×100%
Data represent the mean values ± SE (n = 3)
Different letters represent significant differences

Table 2. Effect of 1/2MS medium fortified with different concentrations of NAA on root induction.

No.	NAA (mg/L)	Number of shoots cultured	Percentage of roots induction (%)
B1	0.1	50	97.33 ± 0.88^{c}
B2	0.2	50	71.33 ± 2.91^{b}
B3	0.3	50	56 ± 7.00^{b}
B4	0.4	50	26.67 ± 5.70^{a}

Percentage of roots induction (%) = the number of roots of shoots/the number of shoots × 100%
Data represent the mean values ± SE (n = 3)
Different letters represent significant differences

Molecular analysis of transformants: Genomic DNA isolated from surviving transformed and non-transformed plantlets was used as PCR template. The plasmid pRI101-Mgfp was used as positive control, while the wild-type plant and the plant of the LBA4404 strain that harbors the parent vector pRI101-AN were used as negative control. PCR assay showed that the specific 260-bp band of the *Mgfp-5* gene was amplified in several of the putative transformed shoots, but not in non-transformed plantlets and negation control (Fig. 3A). Additionally, RT-PCR analysis was performed to amplify a 260-bp band (Fig. 3B). The transcript of the *Mgfp*-5 gene was detected in the transgenic plants, but not in the non-transformed plants.

The highest transformation efficiency of 3.33% was obtained from precultured explants immersed in an *Agrobacterium* infective suspension with an $O.D._{(600)}$ of 0.6 (Fig. 4A). The use of *Agrobacterium* infective suspensions with higher densities resulted in decreased transformation efficiencies due to increased infection (Table 3). Different combinations of two factors were tested: the $O.D._{(600)}$ value of the bacterial suspension and the infection time. We tested the immersion of leaf discs

for 5, 10, and 15 min in *Agrobacterium* suspension solutions at $O.D._{(600)}$ values of 0.4, 0.6, and 0.8. The highest transformation efficiency of 9.67% was obtained when the leaf explants were inoculated with *Agrobacterium* for up to 10 min at an $O.D._{(600)}$ of 0.6 (Table 3). With an immersion time of over 10 min or an $O.D._{(600)}$ value of 0.8, overgrowth of *Agrobacterium* was observed on the surface of the leaf disc, leading to necrosis. Additionally, the duration of cocultivation of the *Agrobacterium* infective suspension and explants was an important factor for the transformation efficiency. The results of our study suggest that cocultivation at 3 d produced the highest transformation efficiency of 2.67%, with significant differences in efficiency between the tested times (Fig. 4B).

Screening of transgenic plants progeny: Seeds from T_0 transgenic plants and untransformed control plants were germinated on MS containing 50 mg/L Kan. Both could germinate and few plants had bleached with the passage of time (Fig. 5A, B). However, non-albino transgenic seedlings had grown into plantlets, while all untransformed plants had died after about two months (Fig. 5C, D). The surviving T_1 seedlings form T_0 transgenic plants were PCR screened for the trans-gene and the segregation of the trans-gene was tracked. The results showed the expression of *Mgfp*-5 gene by amplification of the expected a 260-bp fragment and the segregation of the *Mgfp*-5 gene in T_1 generation transgenic plants #9 and #19 at a ratio corresponding to the expected 3:1 Mendelian segregation ratio obtained via X^2 analysis (Fig. 3C, Table 4).

Once T_1 transgenic plants #9 and #19 expressing the *Mgfp*-5 gene were obtained, the presence of Mgfp-5 protein was verified via Western blot analysis using the anti His-Tag mouse monoclonal antibody. As shown in the Western blot analysis, plants #9-1, #9-2, and #19-1 showed specific band sized of about 11 kD, while the wild-type plant and plant #19-2 showed no such band (Fig. 6). This showed that the gene *Mgfp*-5 was not expressed in plant #19-2. These results indicate that the Mgfp-5 transgenic chicory was obtained with *Mgfp*-5 gene expression.

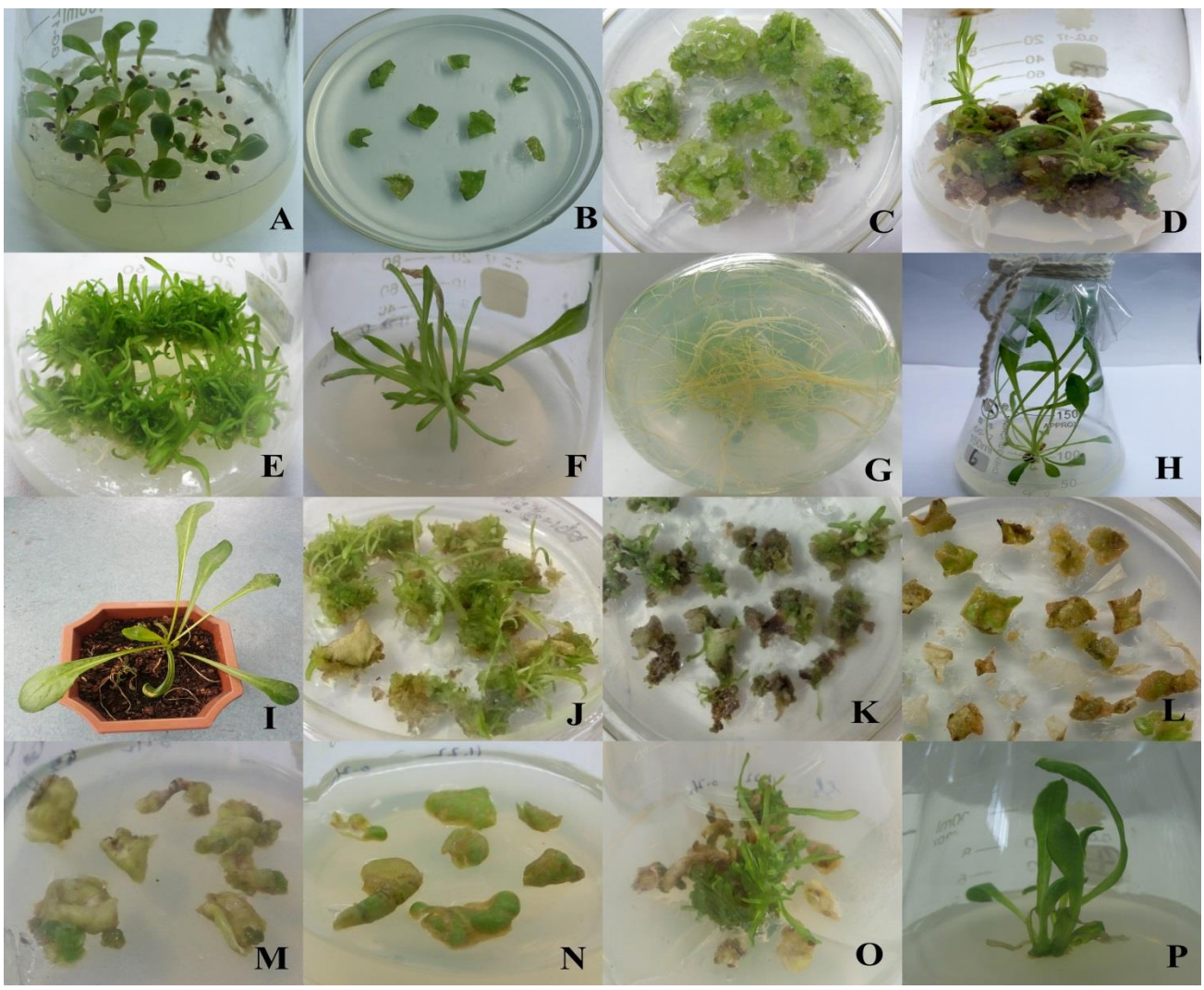

Fig. 1. Callus and shoots regeneration of chicory
(A) Seed germination on MS medium. (B~F) Callus and shoots induction on the optimum CSM, (D) Green shoots of Browning callus tissue. (G) Roots induction on 1/2MS+0.1 mg/L NAA medium. (H) Rooted plantlets,(I) Well-rooted plantlet grown in the soil, (J-M) surviving of uninfected leaf segments cultured on different concentration of Kan, (N) Screening of putative transformants CSM (contained 50 mg/L Kan), (O)Survival transformed plant on 50 mg/L Kan, (P) Root induction on 50 mg/L Kan.

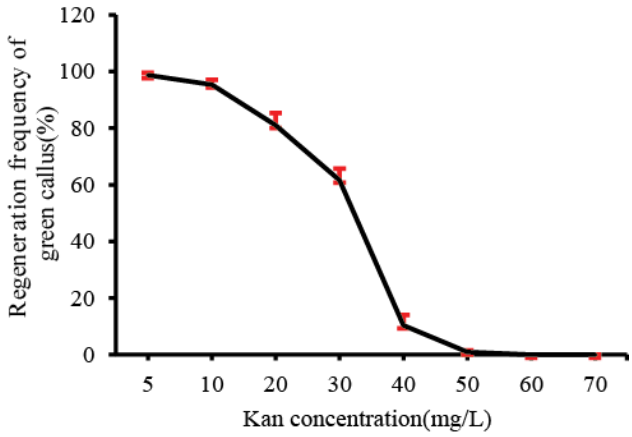

Fig. 2. Determination of kanamycin concentration for putative transformants selection
Regeneration frequency of green callus (%) = the number of regenerated callus/the number of leaf explants × 100%
Error bars represent standard error (n=3)

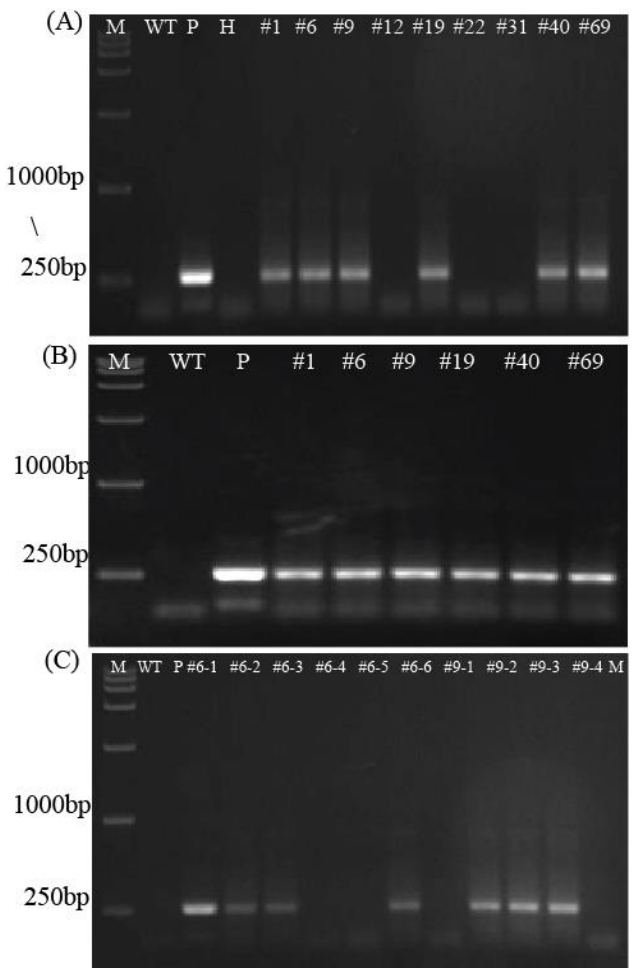

Fig. 3. Polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR) analyses transgenic chicories
M, DNA Marker; WT, wild-type plant; P, plasmid pRI101-Mgfp; H, the plant of the LBA4404 strain that harbors the parent vector pRI101-AN (A) PCR analysis of putative T_0 transformants showing amplification of 260-bp fragment of *Mgfp*-5 gene and vector. (B) RT-PCR analysis of *Mgfp*-5 and vector positive T_0 plants, showing amplification of 260-bp fragment of *Mgfp*-5 gene. (C) PCR analysis showing the integration of the 260-bp *Mgfp*-5 trans-gene into the primary transformant #6, #9 and its segregating T_1 progenies.

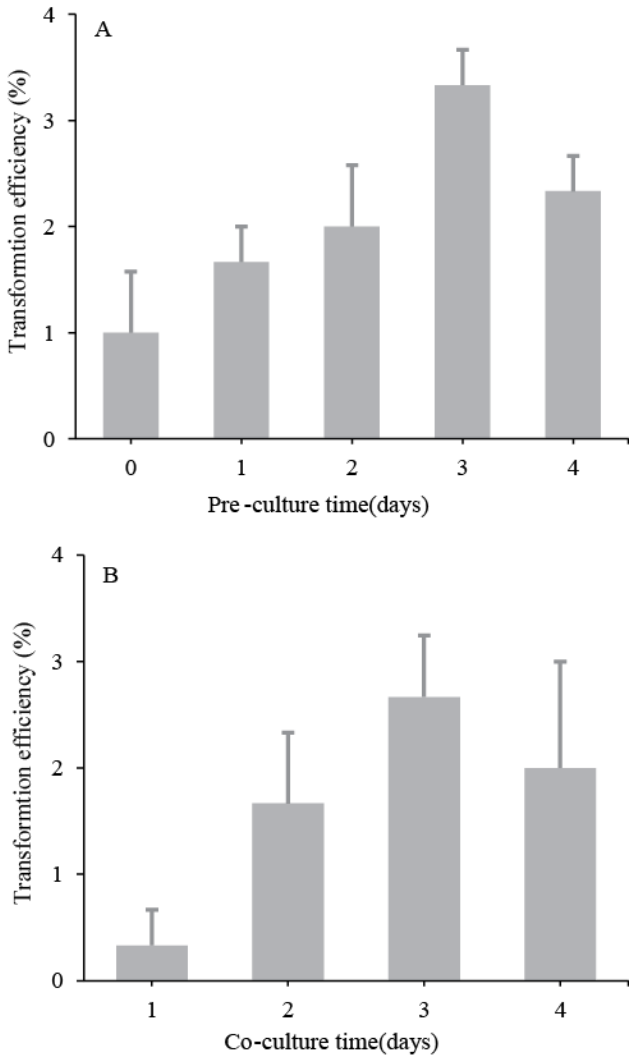

Fig. 4. Effects of pre-culture and co-culture period on the rate of differentiation
Transformation frequency (%) = the number of PCR-positive plants/the number of shoot evaluated. Data represent the mean values ± SE (n = 3).

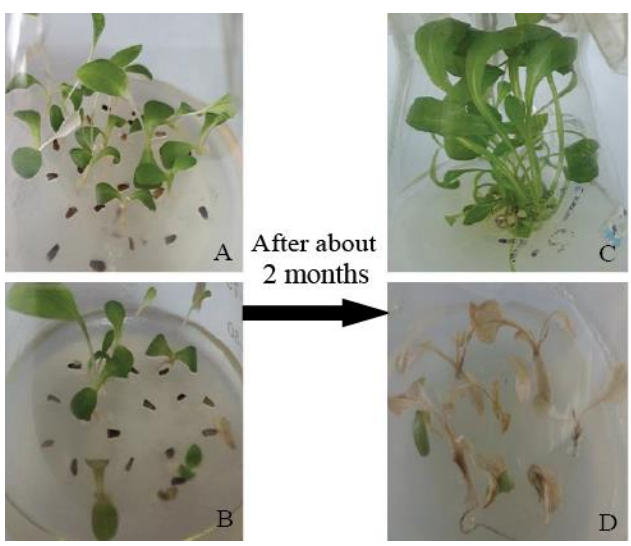

Fig. 5. T_1 seeds from transgenic plants and untransformed plants germinated on medium containing 50 mg/L Kan
(A, C) T_1 transgenic plantlets. (B, D) T_1 untransformed plantlets.

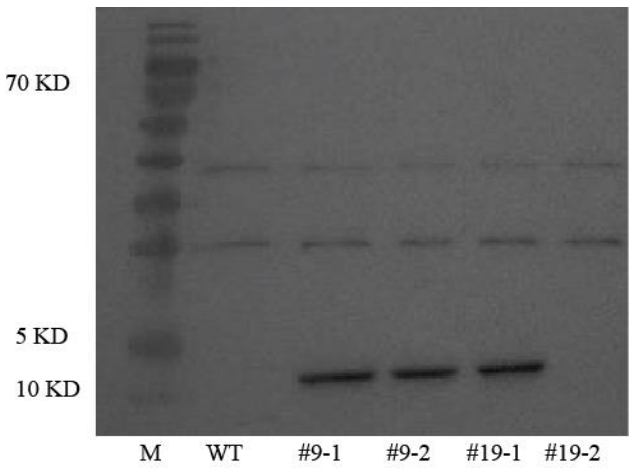

Fig. 6. Western blotting analyses of T_1 transgenic plants
It showed the specific bands (about 11 KD) of the protein Mgfp-5. M, protein Marker; WT, wild-type plant; plant #9-1, #9-2 and plant #19-1, #19-2

Discussion

Plant growth regulators play a key role in the *In vitro* regeneration of plants. The combination of 6-BA and NAA has been widely used for callus and shoot regeneration from leaf explants in various plant species (Dhar & Josh, 2015, Zeng and Zhao, 2015). In the same way, the combination of different proportions of 6-BA and NAA are often used in chicory (Maroufi 2015). In our current study, inoculation on 1.5 mg/L 6-BA and 0.2 mg/L NAA has shown the best response with a regeneration frequency of 79.18%. Zhao *et al*., (2012) reported that salt concentration of half of the 1/2 MS medium worked better than the MS medium rooting effect for the induction of roots. During this experiment, the 1/2 MS medium was selected and roots were successfully induced after the regeneration of shoots. Therefore, the optimal medium was 1/2 MS containing 0.1 mg/L NAA for root induction.

Elucidating the effects of Kan on the growth of different species plants at various concentrations is a preliminary task of transformation research. Antibiotic resistance studies include Kan and hygromycin selection marker, included in the vector along with the goal genes. This offers a convenient and efficient selection system. With regard to the sensitivity to antibiotics in plants, the selection is very low (Cheng *et al*., 1996, Sharma & Anjaiah, 2000). However, most plants (including chicory) can be successfully selected on Kan-based selection systems (Rommens 2006, Oliveira *et al*., 2011, Mishra *et al*., 2013, Alvarez & Ordás, 2013, Frulleux *et al*., 1997, Matvieieva *et al*., 2011). Leaf explants were highly sensitive to increasing concentrations of Kan, and the sensitivity symptoms included bleaching, shortening of leaves, stunted growth, and a varying degree of yellowing, as earlier reported by Saha *et al*., (2014). When the Kan concentration is too high, it showed strong toxicity to cells and quickly killed many explants; In contrast, when the concentration is too low, it produced putative resistant buds and many chimeras (Cheng *et al*., 2004, Joersbo & Okkels, 1996). In this study, the concentration of 50 mg/L Kan has been successfully used for the selection of transformed plants in chicory.

For the genetic transformation of plants, the pre-culture of leaf explants can promote cell division, which helps *Agrobacterium* infection and aid the integration of exogenous DNA, thus enhancing transformation efficiency (Birch 1997, Bullea *et al*., 2015). This was likely due to the likeliness of T-strand integration into cells that actively underwent cell cycle progression (Villemont *et al*., 1997). Pre-cultured explants are beneficial for the *Agrobacterium* infection and rotting browning reduction, and further reduce damage to the explants while adjusting the cell physiological condition. However, the pre-culture period is too long, which can cause wound healing and rendering plants unfit for *Agrobacterium* infection. In this study, pre-culture of 3 d was found to be optimal for transformation. Pre-culture period shorter or longer than 3 d reduced the transformation efficiency.

Agrobacterium cell concentration and infection time are also important factors influencing the genetic transformation efficiency (Khan *et al*., 2015). Lower *Agrobacterium* density or shorter infection time cannot guarantee sufficient gene transfer, while higher *Agrobacterium* concentration or longer infection time could harm the plant cells and result in browning and even death of the explants.

Co-culture is a key step of the *Agrobacterium*-mediated conversion containing *Vir* genes induction, T-DNA transfer, and T-DNA integration, and co-culture can also directly affect the conversion efficiency. Thus, if the duration of co-cultivation was too short, transference of T-DNA would not be completed. If the exposure was too long, the eugenic Agrobacterium damage the plant cells and inhibited their regeneration (Hiei *et al*., 1994; Smith & Hood 1995). The results of our study suggest an $O.D._{(600)}$ = 0.6 exposure for 10 min and co-culture for 3 d as ideal for the genetic transformation. In this study, the T_0 generation transformed chicory plants were successfully selected with a mean stable transformation frequency of 9.67% using Kan-based selection systems.

The aim of this study was to increase the probability of stable transformation by optimizing various transformation conditions. However, to show whether the target gene would show stable inheritance and expression correctly, a large number of offspring plants has to be analyzed. Therefore, we obtained T_1 generation seeds from the T_0 generation of transformed chicory plants and found that the majority of progeny plants have appeared following Mendelian inheritance 3:1. Moreover, progeny plants inherited and stably expressed the transgene without exhibiting any morphological aberrations. Our expressed recombinant Mgfp-5 had a His_6 tag, and thus we analyzed next using Western blotting. The molecular weight of the recombinant protein Mgfp-5 is 9.8 kD, which may be because the Mgfp-5 proteins isoelectric point (9.8) is higher in combination with SDS (Hwang *et al*., 2005). Consequently, the molecular weight of the Mgfp-5 protein increased during SDS-PAGE. This indicated that the gene *Mgfp*-5 was expressed in chicory. A previous study reported the expression of the recombinant Mgfp-5 in *E. coli*, which provides a very efficient system for the Mgfp-5 production. However, a subset of the recombinant Mgfp-5 existed as a dimer and had been degraded. However, we did not find a dimer in the transformed chicory. The reason may be that molecular farming in plants is a better system than in *E. coli*.

To the best of our knowledge, this is the first report of *Agrobacterium*-mediated stable genetic transformation in chicory depicting stable inheritance of the transgene by the progenies. This study will promote further research on plant gene engineering and biomedical adhesives.

Table 3. Effects of different infection time and bacterial density of *Agrobacterium* strain LBA4404 on transformation efficiency of Chicory.

No.	O.D.$_{(600)}$	Infection time (min)	Percentage of callus induction (%)	Transformation efficiency (%)
1.	0.4	5	32. 67 ± 7.09^{d}	0.33 ± 0.33^{a}
2.	0.4	10	25.67 ± 4.51^{cd}	2.33 ± 0.88^{a}
3.	0.4	15	16.67 ± 6.43^{abc}	6.67 ± 1.20^{bc}
4.	0.6	5	23.67 ± 4.16^{cd}	4.67 ± 0.88^{b}
5.	0.6	10	19.33 ± 5.03^{bc}	9.67 ± 0.67^{d}
6.	0.6	15	9.67 ± 2.33^{ab}	7.00 ± 0.58^{c}
7.	0.8	5	19.00 ± 7.21^{bc}	5.67 ± 0.33^{bc}
8.	0.8	10	11.67 ± 3.06^{ab}	2.00 ± 0.58^{a}
9.	0.8	15	8.33 ± 2.08^{a}	0.67 ± 0.33^{a}

Percentage of callus induction (%) = the number of callus/the number of leaf explants × 100%
Transformation frequency (%) = the number of PCR-positive plants/the number of shoots
Data represent the mean values ± SE (n = 3)
Different letters represent significant differences

Table 4. Segregation analysis of *Mgfp*-5 gene in T_1 generation progeny plants.

Plant ID	Total seedlings tested	*Mgfp*-5 positive	*Mgfp*-5 negative	*Mgfp*-5^{+}/ *Mgfp*-5^{-}	Best fit segregation ratio	X^2 value	*P* value
#6	51	28	23	1.22	1:1	0.49	0.49
#9	54	43	11	3.90	3:1	0.62	0.45
#19	49	35	14	2.50	3:1	0.33	0.59
#40	60	39	21	1.85	3:1	4.32	0.04

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