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Expression of human granulocyte-colony stimulating factor (*hG-CSF*) gene in tobacco (*Nicotiana tabacum*)

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Abstract

Granulocyte colony-stimulating factor (G-CSF) is a member of the CSF family that regulates hematopoietic cell proliferation and differentiation. In this research, Tobacco (*Nicotianatabacum* L., var. NC-2512) leaf disc explants were transformed with *Agrobacterium tumefaciens* (LBA4404 strain), harboring the recombinant binary vector pBI121 containing *hG-CSF* gene and neomycin phosphotransferase (*nptII)* antibiotic resistant gene. Inoculated tissues (leaf discs) were placed on tobacco co-cultivation medium and then transformed explants were selected on MS medium containing 50 mgl^{-1} kanamycin and 300 mgl^{-1} cefotaxime antibiotic combinations. Finally, shoots were transferred to hormone free medium containing 50 mgl^{-1} kanamycin and 200 mgl^{-1} cefotaxime antibiotics to induce roots. Polymerase chain reaction using specific primers was employed to confirm the integration of *hG-CSF* and *nptII* transgenes in the T_0 and T_1 plants genome. However, merging of *hG-CSF* gene into the genome of putative transgenic plants was further verified by Southern blot analysis. In addition, molecular analysis was performed at the mRNA and protein levels. In the first transgenic tobaccos (T1), the mRNA transcripts were analyzed by RT-PCR method which strongly showed presence of *hG-GCF* transcript. Ultimately western blot analysis of T_2 plants approved stable transformation and expression of *hG-CSF* gene in transgenic plants. The χ^2test of T_1 plants in greenhouse condition indicated that the inheritance of *hG-CSF* gene followed Mandelian ratio for single gene segregation (3:1).

Keyword: *Agrobacterium tumefaciens, hG-CSF,* tobacco, transformation, T_2 plants.
Abbreviations: BAP-Benzylaminopurine**;** MS media-Murashige and Skoog**;** NAA-Naphthalene acetic acid; TBST -Tris Buffer Saline

Introduction

The need for recombinant therapeutic molecules for clinical application has been increased extremely over the past decades. Apart from the microbial and animal cell culture systems, recent progress provided a rich source for various bioactive molecules by using plants as bioreactors to produce recombinant proteins (Obembe et al., 2011; Miao et al., 2008). Also the development of new expression system for cost-effective production of pharmaceuticals has become significantly important (Giddings, 2001; Giritch et al., 2006; Miao et al., 2008; Rajabi Memari et al., 2010). Granulocyte colony-stimulating factor (G-CSF) consisted of various products which are derived from different tissues, stimulate the bone marrow to produce granulocytes following by releasing them into the blood (Franzke**,** 2006; Chang and Huang, 2011). Traditional production systems that use microbial fermentation, insect and mammalian cell cultures, and transgenic animals, have drawbacks in terms of cost, scalability, product safety and authenticity (Houdebine, 2000; Swartz, 2001; Chu and Robinson, 2001). Recent studies have shown that molecular farming in plants has many practical, economic and safety advantages compared with other conventional systems, and also the use of plants for large-scale protein synthesis is gaining wider acceptance (Fischer and Emans, 2000; Giddings, 2001; Huy et al., 2011; Bock and Warzecha, 2010). Plants have several advantages as a bioreactor; they are highly scalable, capable of producing biologically active compounds, eukaryotic PTM (post-translational modification) and free of mammalian viral and pathogens (Karg and Kallio, 2009). The mature human G-CSF is a 19.6 kDa glycoprotein with 174 amino acids (Nagata et al., 1986; Souza et al., 1986). Naturally, there are two different forms of GCSF native protein, type (a) and type (b) which (b) is more active than type (a) (Asano, 1991). Because of that in current research, the type b was cloned and expressed with 174 amino acids. Nevertheless, the aims of this study were to transform and explicit the active form (type b) of Neupogen drug (G-CSF recombinant protein) in plant tobacco for further therapeutic consideration.

Table 1. Stability of *hG-CSF* gene in different transgenic events as judged by their transmission to the next generation. The table summarizes the number of PCR positive (*hG-CSF*$^{+}$) and negative (*hG-CSF*$^{-}$) T_1 plants

Line	*hhhG-CSF*$^{+}$	*hG-CSF*$^{-}$	Total
T_{1A}	21	10	31

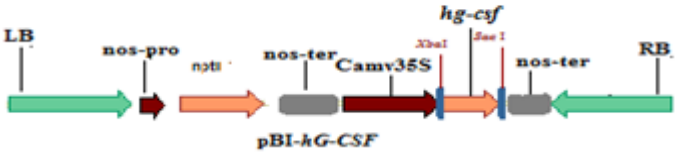
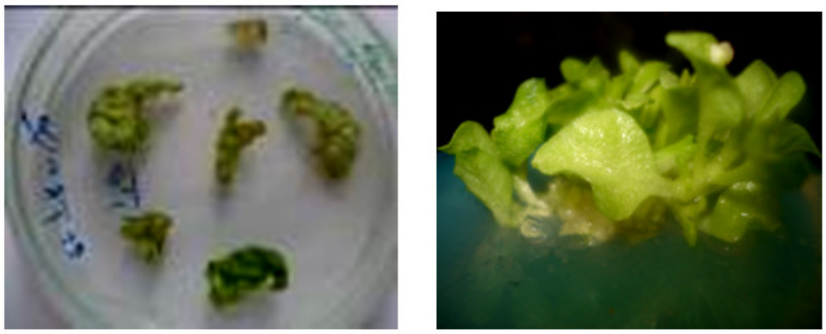


Fig 1. Schematic map of the pBI-*hG-CSF* vector. LB, left border; RB, right border; Nos-P/T, nopaline synthesis promoter/terminator sequence; 35S promoter, cauliflower mosaic virus 35S RNA promoter sequence; NPTII, neomycin phosphotransferase type-II.

Table 2. The Chi-Square test for determining transmission ratio and inheritance of *hG-CSF* gene in T_1 generation.

X	O_i	E_i	(O_i-E_i)	$(O_i-E_i)^2$	$(O_i-E_i)^2/E_i$
3	21	24	-3	9	0.65
1	10	7	3	9	0.25
Total	31	31	-	-	0.86

O_i:: Observed data, E_i: Expected data, X: Ratio of inheritance.



A B

Fig 2. Different regeneration stages of the transgenic tobacco plants: (A), Explants on regeneration medium containing antibiotics (50 mgl^{-1} kanamycin and 300 mgl^{-1} cefotaxime). (B), 4-5 week tissues showing regeneration of adventitious shoot.

Results

Transformation and effective regeneration system

Three weeks after transformation, shoot regeneration was observed on MS medium supplemented with kanamycin (50 mgl^{-1}) and cefotaxime (300 mgl^{-1}) (Fig 2, A). The T-DNA of the transforming vector pBI-*hG-CSF*, including the *nptII* marker gene (Fig 1), was integrated into the chromosomal genome of plant cells, so that only transformed cells were survived in the medium supplemented with kanamycin (Fig 2, b). To set up an effective regeneration system suitable for *Agrobacterium*-mediated transformation, it was necessary to determine the minimal concentrations of kanamycin and cefotaxime in the differentiation medium. Within 2 months of culture, untransformed control explants on selection medium (50 mgl^{-1} kanamaycin) turned yellow. The kanamycin resistance explants accompanied by *nptII* gene was also successfully used as a marker, because a short period of kanamaycin treatment (50 mgl^{-1}) in the selection medium effectively blenched many non *nptII* expression shoots. The minimal concentration of kanamycin (50 mgl^{-1}) to select the transformed tissue was estimated by counting the necrotic explants on MS medium containing various concentrations of kanamycin. Adding more cefotaxime showed no toxicity to tobacco tissues even at a concentration of 300 mgl^{-1} (Fig 2, B).

Molecular analysis of transgenic plants

Recombinant hG-CSF gene presence and inheritance pattern analysis

The PCR analysis was carried out to confirm the presence of the *hG-CSF* and *nptII* genes in the genome of the putative transgenic explants (Fig 3 and 4). The results for *hG-CSF* gene on 31 T_1 generations showed that, 21 out of 31 T_1 plants were positive by polymerase chain reaction (Fig 3 and table 1). Furthermore, data analysis by Chi-Square distribution test confirmed 3:1 segregation ratio (table 2). The ~500 bp expected *hG-CSF* fragment was found in the positive control (pBI-*hG-CSF* plasmid) as well as in the assumed transgenic plants. Untransformed plants (controls) did not show the fragments. The same results were obtained for ~900 bp for

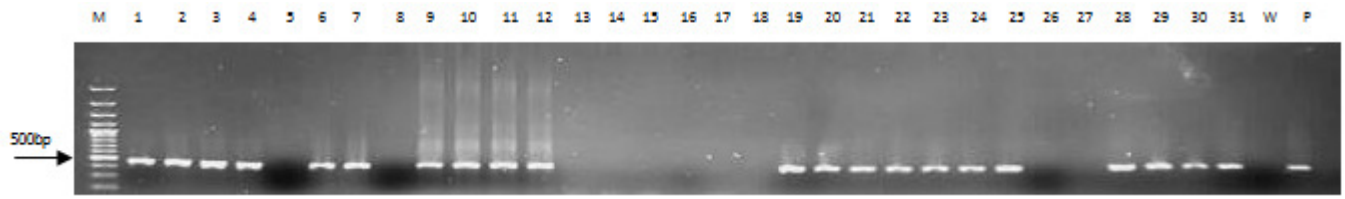


Fig 3. PCR analysis of DNA isolated from leaves of transformed tobacco using specific primers for amplification of the 500bp *hG-CSF* gene on agarose gel. M, 1.0 kb plus DNA ladder (Gibco BRL). W, non transgenic plant. P, plasmid (pBI121-*hG-CSF)*. 1-31, present and absent of *hG-CSF* in T_1 transgenic lines

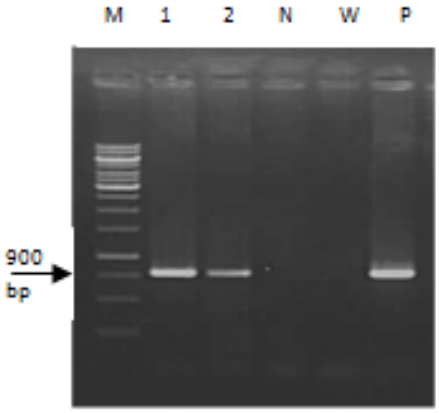


Fig 4. PCR analysis of DNA isolated from leaves of transformed tobacco using a pair of specific primer for amplification of the 900 bp *nptII* gene on agarose gell. M, 1.0 kb plus DNA ladder (Gibco BRL). 1 and 2, present of *nptII* in T_1 transgenic lines. N, non transgenic plant. W, water (no DNA) P, positive control (pBI121_*nptII*).

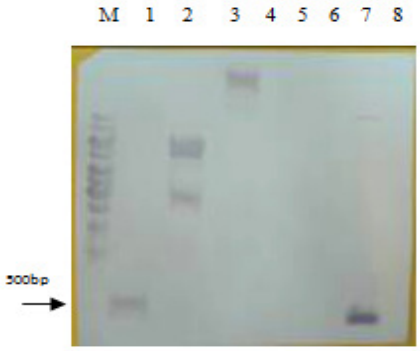


Fig 5. Southern blot analysis of DNA isolated from leaves of T_1 transgenic tobacco**:** M, 1Kb plus DNA ladder. 2, double digested DNA extracted from transgenic tobacco with both of *Xba*I and *Sac*I restriction enzymes 3, *xba*I digested DNA extracted from transgenic tobacco 4, undigested DNA extracted from transgenic tobacco. 5, double digested DNA extracted from non-transgenic tobacco with both of *Xba*I and *Sac*I restriction enzymes. 6, *xba*I digested DNA extracted from non-transgenic tobacco 7, plasmid: double digested pBI-*hG-CSF* with both of *Xba*I and *Sac*I restriction enzymes 8, undigested DNA extracted from non-transgenic tobacco.

nptII gene in the genome of the presumed tobacco transgenic samples.

Southern blot

Southern blot analysis showed that the hybridization of probe to the undigested DNA occurred exclusively at a high molecular weight, indicating the integration of the gene into the tobacco genome. Most of the hybridizations were appropriated to an anticipated fragment of ~500 bp including the whole coding sequence of the *hG-CSF* gene when the DNA were digested with *Xba*I and *Sac*I. This result showed that there is at least one copy of the joined *hG-CSF* gene in the genome (Fig 5). Only two bands of different size were detected in each of the different transgenic lines when the digested DNA with *Xba*I was hybridized to the probe. This result indicated the presence of only two copies of the transgene in one of the tested events, since there is only one *XbaI* site in the T-DNA (Fig 1). No hybridization signal could be discerned for the DNA extracted from untransformed plants as shown in (Fig 5).

RT-PCR analysis

T_1 plants expression profiling was studied by RT-PCR. The analysis was performed with RNA samples isolated from independent transgenic and control plants. The results pointed out the presence of 522 bp band as expected (Fig 6). All the PCR positive plants did not show the same results for RT-PCR. Only five out of eight plants were positive by RT-PCR. RNA template along with Taq DNA polymerase was used in control reaction to make sure there is no genomic DNA pollution in RNA pool (Fig 6). Moreover, no signal was observed for RNA extracted from untransformed plants.

Expression of hG-CSF gene in T_2 transgenic tobacco

The T_2 transgenic plants were analyzed for *hG-CSF* expression using western blot analysis. Three out of four transgenic plants showed a protein band of expected size (20 kDa) which was the result of protein reaction with G-CSF antibody (Fig 7).The level of *hG-CSF* expression varied among the transgenic plants. Untransformed control plant did not show any positive band for the *hG-CSF* protein.

Discussion

Traditional recombinant protein production systems like bacterial and yeast fermentation, transgenic animals and

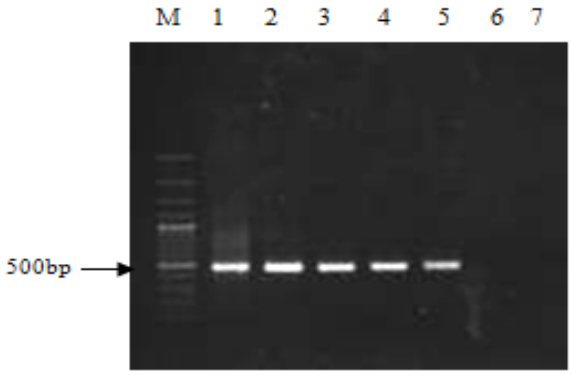


Fig 6. :RT-PCR analysis of total RNA of T_1 plants to detect the transcript of *hG-CSF* gene. M: 1kb size marker (fermentase). Lanes 1-5: T_1 plants showing 500 bp. *hG-CSF* cDNA; Lane 6: RNA of T_1 plant used as template without RT-reaction. Lane 7: control (Amplification of RNA template by TaqDNA polymerase).

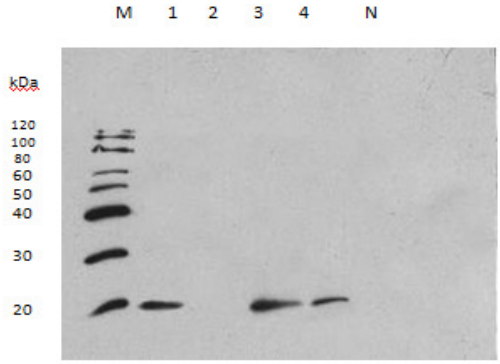


Fig 7. Expression of *hG-CSF* gene in T_2 transgenic tobacco plants. Protein immunoblot to detect *hG-CSF* protein (20 kDa) in total soluble leaf protein. lane M: protein marker (MagicMark™ XP Western Protein Standard, Invitrogene). Lanes: 1–4, transgenic lines. Lane N: non-transgenic plant as negative control.

mammalian cell cultures have limitations in safety, cost, scalability etc. Therefore, inexpensive and simple platform that can produce safer, cheaper and high level recombinant proteins would be demanded. Plant expression systems have several characteristics for production of therapeutic recombinant proteins; elimination of the potential contamination of endotoxins and animal viruses which are common problems in bacterial and eukaryotic expression systems (Obembe et al., 2011; Park and Cheong, 2002). Reports have shown successful expression of biologically active human proteins in plant expression systems (Hong et al., 2006; Huy et al., 2011; Bock and Warzecha, 2010). Transgenic plant expression systems have some special advantages compared to prokaryotic expression systems, For example, Rajabi Memari et al. compared *E-coli* expression system with plant expression system and they have shown that plant produces high quality recombinant proteins. It has been suggested that the post translational modifications such as disulfide bond formation completely is carried out in plant (Lombardi et al., 2009; Orsi et al., 2001). In contrast, in prokaryotic system, disulfide bond formation mainly occurs in the periplasmic space (Sevier and kaiser, 2002; Witt, 2008). Large scale protein production is a major advantage of using transgenic plants in the recombinant protein industry (Obembe et al., 2011). Using transgenic plants reduces skilled personnel required for running complex equipment such as bioreactors, so the capital and running costs are significantly lower compared to bacterial and animal cell-based production systems (Ma et al., 2005; Twyman et al., 2005). The rhG-CSF generated global sales of $5.6 billion (June 2005 to June 2006) and its market in Europe and USA has the potential to generate sales of approximately $605 million in 2010 (Pisani and Bonduelle, 2006). Two types of G-CSF are clinically available: a glycosylated form of a recombinant therapeutic agent (lenograstim), which is expressed in mammalian cells, and nonglycosylated form (filgrastim), which is produced by using *E- coli* expression system (Vanz et al., 2008). In current study, we produced glycosylated form of G-CSF which can be more effective than non-glycosylated form that is currently produced in prokaryotic systems and is used in cancer chemotherapy. The plant expression vector pBI121 was used in plant transformation. This vector carries kanamycin resistance gene for prokaryotic selection as well as another kanamycin resistance gene for the eukaryotic screening. The transgene inserted between CaMV35s promoter and NOS terminator in T-DNA region. In dicot plants CaMV35S is a suitable promoter because it is potent and constitutive and it can cause high-level expression in leaves, roots and seeds. The *hG-CSF* gene integration into the nuclear genome of T_0, T_1 and T_2 plants was confirmed by PCR and southern blot analysis. Expression of recombinant *hG-CSF* gene in transgenic plants was also showed by RT-PCR and western analysis, however, some of the transgenic lines that were detected by PCR and southern blot analysis did not confirmed by expression analysis, that possibly resulted from low levels of transcription and translation and it can also occur due to gene silencing events (Metzlaff et al., 1997). The growth rates and flowering were not affected in transgenic tobacco. In addition, when germinated on the medium containing 50 mgl^{-1}kanamycin T_2 seedlings were observed as green (data not shown). T_2 molecular analysis confirmed that human *hG-CSF* gene has been stably inserted and inheritably expressed in tobacco genome. In fact, the recombinant gene transcription and translation have been processing successfully in tobacco cells. In summary, we effectively examined the cloning and expression of *hG-CSF* gene in tobacco. Since there are different types of recombinant proteins which are being tested in clinical trials (http://www.moleculartarming.com), we hope that the *hG-CSF* gene products could serve as a valuable reagent for studies of the human blood cancer.

Materials and methods

Plant transformation vector construction

Standard cloning techniques were used to construct the recombinant plasmid (Sambrook and Russell, 2001). The *hG-CSF* cDNA cloned in pBluescriptIISK (-) was obtained from Institute of Biotechnology, Malek-E-Ashtar university. It was digested with *BamH*I and *Hind*III restriction enzymes and then cloned into the same restriction sites of *lacZ*$^-$ operan of pGEM7Zf (-). Subsequently, the *hG-CSF* gene digested with

*Xba*I and *Sac*I and inserted in homologous sites of pBI121 binary vector (PBI-*hG-CSF*). Target gene expression was under direct control of CaMV35S promoter of cauliflower mosaic virus and NOS terminator (Fig 1). The binary vector construct carrying the *hG-CSF* gene was transformed to *Agrobacterium tumefaciens* strain LBA4404 by freeze and thaw method (An, 1987).

Plant materials

Seeds of tobacco (*Nicotiana tabacum* L., var. NC-2512) were surface sterilized with 70% ethanol for 1 min followed by 30 min in 2.5% sodium hypochlorite. Seeds were then germinated on ½ MS medium and grown in an environmentally controlled culture room at 25°C on a photoperiod of 16 h light and 18 h dark. Leaf tissue from 1 month culture was used as an incubation source with *Agrobacterium* suspension.

Co-cultivation, selection and plant regeneration

Leaf discs ($1cm^2$) derived from cultured seeds were used for transformation. The *Agrobacterium* strain LBA4404 harboring *pBI-hG-CSF* was grown overnight. The bacterial cells were solid as a pellet and resuspended in 50 ml of MS medium without sugar (pH= 5.8) and grown for 6 h to reach to appropriate OD. The leaf discs were soaked in the bacterial suspension for 20 min, and then were transferred on the co-culture medium. Co- cultivation was carried out at 25°C in the dark on the MS medium supplemented with NAA (0.1 mgl^{-1}) and BAP (2 mgl^{-1}) for 2 days. Subsequently, the leaf discs were transferred on the MS medium supplemented with 0.1 mgl^{-1} NAA, 2 mgl^{-1} BAP, 50 mgl^{-1} kanamycin and 300 mgl^{-1} cefotaxime for selection and regeneration of transformed explants (Hong et al., 2006). The explants were transferred onto the same fresh medium after 2 - 3 weeks (Hong et al., 2002). The well-developed shoots were transferred to hormone free medium containing, 50 mgl^{-1} kanamycin and 200 mgl^{-1} cefotaxime for rooting. After several months the transgenic seeds (T_1) were collected and cultured on MS medium containing 50mg/l kanamycin. Thirty one of T_1 plants were analyzed by PCR (Fig 3) and some of them were used for southern blot hybridization (Fig 5). The PCR and southern blot positive plants were selected and transferred into the pots in greenhouse to achieve T_2 plants. Eventually, some PCR positive T_2 plants were selected for performing western blot analysis (Fig 7).

PCR and statistical analysis of transgenic plants

Genomic DNA was isolated from young leaves of regenerated plants by using the modified CTAB method (Huang et al., 2000). The PCR analysis carried out for the *hG-CSF* and *nptII* genes. Primers sequences which were designed for a ~500 bp of *hG-CSF* and 900 bp of *nptII* were as: *hG-CSF* forward (5'-CCTGCTCAAGTGCTTAG-3'), *hG-CSF* reverse (5'-TAGAACGCGGTACGAC-3') primers, *nptII* forward (5'-GAACAAGATTGCACGC-3') and *nptII* reverse (GAAGAACTCGTCAAGAACGC). In order to characterize *hG-CSF* gene inheritance pattern, the polymerase chain reaction was performed for 31 T_1 plants (table 1). The PCR was initiated by a hot start at 94°C for 5 min followed by 35 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C, and a final extension step at 72°C for 5 min (Fig 3). Consequently, the chi-square method was used for (O_i) comparing observed counts to the (E_i) expected amounts (table 2).

Southern blot analysis

Genomic DNAs were extracted from T_0 and T_1 plants, according to the cetyltrimethyl ammonium bromide (CTAB) method (Huang et al., 2000). The PCR-positive plants and the untransformed plants were analyzed by Southern blot analysis to confirm the integration of the introduced gene. Two separate digested genomic DNA were set up for each plant sample, using 30µg of DNA for both experiments. The *Xba*I restriction enzyme was used to determine the number of joined *hG-CSF* fragments into the genome of transformed plants. Moreover, both *Xba*I and *Sac*I (550 bp) enzymes were used to determine whether or not the complete *hG-CSF* gene had been integrated into the plant genomes. After separation of digested and also uncut DNA, they all were transferred onto a HybondTM N membrane (Roche Co. Ltd, Germany), (Fig 5). Finally hybridizing, washing and detection procedures performed following the manufacturer's instruction (Roche Co. Ltd, Germany).

RT-PCR analysis

Reverse transcription (RT)-PCR amplification was performed with SuperScriptIII® One-Step RT-PCR kit (Invitrogen, CA), according to the manufacturer's instructions. Total RNA was isolated using Trizol method (Kansal et al., 2008). After isolation, total RNA was treated with RNase free DNase (Invitrogen, CA) to ensure the complete removal of genomic DNA. One µg of purified total RNA was used in each RT-PCR amplification reaction. The same primers which utilized for PCR were also tested for RT-PCR. The limited cycle PCR was consisted of 2 min at 94°C, 40 cycles of 30 s at 94 ºC, 30 s at 60 ºC, 1 min at 68 ºC, and 1 cycle of 7 min at 68 ºC.

Protein extraction, Western blot analysis

Three hundred mg of leaf samples from transgenic and control plants were grounded to fine powder and added to 300 µl extraction buffer consisting of 100 mM Tris–HCl and 50 mM 2-mercapthoethanol. Protein concentration was estimated by dye binding method (Bradford, 1976). Seventy five µg soluble proteins from each sample were loaded in a 12% SDS-polyacrylamide gel. After gel electrophoresis, the proteins were blotted to a PVDF membrane (Bio-Rad) by a wet transblotting apparatus following manufacturer instructions (Bio-Rad). The blotted membrane was incubated in TBST (Tris buffer saline with 0.1% Tween-20) containing 5% non-fat dried milk at room temperature for 1.5 h. The membrane was incubated with rabbit anti-G-CSF polyclonal antibody at a dilution of 1:5000 for 6 h at 4°C. subsequently, the membrane was washed three times with TBST for 15 min, and then the membrane was incubated with peroxidase-labeled secondary antibodies against rabbit (1:7.000; Sigma, A2074) for 1 h at room temperature. After three times washings in TBST, the bound of antibodies were visualized by ECL® system (Sigma, CPS-1-120). Finally, the film was scanned with a densitometer (GS-800, Bio-Rad).

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