A. Gatica-Arias, N. Amma, M. Stanke and G. Weber

# *Agrobacterium Rhizogenes*-Mediated Transformation of Hop (*Humulus lupulus* L. cv. Tettnanger): Establishment of a System for Functional Evaluation of Genes*

**Secondary metabolites produced in the lupulin glands (glandular trichomes) of hop cones are important for beer production, and for the pharmaceutical and medicinal industry. However, in wildtype plants the concentration of these compounds is rather low. To enhance their production, it would be interesting to modify by genetic transformation the regulation of genes involved in the biosynthesis of flavonoids and phloroglucinols. Although genetic transformation of hop has been used successfully for some time, it is a laborious and time consuming process. Therefore, the development of a simple and fast assay for functional validation of candidate genes is very desirable. As an alternative *A. rhizogenes*-mediated transformation could be employed for stably expressing genes in hairy roots. The latter being morphologically related to trichomes. *A. rhizogenes* K599 and 15834 were tested for their ability to induce the formation of hairy roots on hop explants. Only *A. rhizogenes* 15834 was able to induce hairy roots after 12-27 days of culture. The highest percentage of explants with roots as well as the total number of roots were obtained when infected leaf segments were cultivated with the adaxial surface in contact with the culture medium. Transgenic hairy roots were verified by GUS staining. The integration of *rolC* and *mgfp5* genes in transgenic hairy roots was confirmed by PCR. We developed a system to investigate the role of genes and transcription factors involved in the biosynthesis of flavonoids and phloroglucinols.**

Descriptors: hop, *Humulus lupulus*, *Agrobacterium rhizogenes*, genetic transformation

## 1 Introduction

Hop (*Humulus lupulus* L., *Cannabaceae*) has been used in the brewing industry and as a medicinal plant. The lupulin glands (glandular trichomes) of the female cones contain $\alpha$-, ß-acids, and essential oils. They are used in beer production as flavoring components, and as preservative agents [1, 2]. Moreover, the lupulin glands contain secondary metabolites (like desmethylxanthohumol, and xanthohumol) with a potential use as pharmaceuticals and in human medicine, due to their anti-cancer, anti-proliferative, antioxidative, and estrogene activity [3, 4, 5, 6, 7, 8]. Therefore, it is of growing interest to develop new hop cultivars with higher content of these important secondary metabolites.

The flavonoid production in plants could be enhanced through genetic transformation with structural genes or by the introduction of genes of homologous or heterologous regulatory elements [9, 10]. For the first time the transcription factor AtMYB75/PAP1 (Production of Anthocyanin Pigment 1), which is a conserved member of the R2R3 gene family, was introduced in *H. lupulus* L. cv. Tettnanger and consequently the production of flavonoids in transgenic clones was elevated [11]. This transcription factor stimulates the expression of genes related to the biosynthesis of phenylpropanoids and flavonoids [12, 13,14].

Successful genetic transformation of hop has been reported using *Agrobacterium tumefaciens*. However, it is a laborious, and time consuming process [11, 15, 16]. Consequently, using *A. tumefaciens*-mediated transformation in hop for functional validation of candidate genes has this major drawback.

Therefore, the development of an efficient, simple and fast transformation protocol for testing gene function in hop is very desirable. Particle bombardment [17] or *A. rhizogenes*-mediated transformation represent alternatives for generating transgenic hop plants. *A. rhizogenes* is a phytopathogenic gram negative soil bacterium responsible for the induction of transgenic hairy

**Authors**

A. Gatica-Arias, N. Amma, M. Stanke, G. Weber, Plant Breeding and Biotechnology, Institute of Plant Breeding, Seed Science and Population Genetics, University of Hohenheim, Stuttgart, Germany

---

* In partial fulfilment of the requirements for a PhD thesis

**Table 1** **Root induction in hop (*Humulus lupulus* L. cv. Tettnanger) after *Agrobacterium rhizogenes* 15834 inoculation of different types of organs**

| Type of inoculated organ | Number of inoculated explants | Explants that produced roots (%) | Total number of roots |
|---|---|---|---|
| Petioles | 100 | 8 | 10 |
| Internodes | 100 | 17 | 40 |
| Leaves-abaxial [a] | 100 | 0 | 0 |
| Leaves-adaxial [a] | 100 | 55 | 126 |

[a] The leaf segments were cultured after the transformation with *A. rhizogenes* 15834::pCAMGFP-CvMV-GWox-Stisy with the abaxial or adaxial surface in contact with the co-culture medium. Data correspond to the experiment 2 (see Material and Methods) and were collected 4 weeks after infection with *A. rhizogenes.*

roots in plants [18]. This agrobacteria transfers a DNA segment (T-DNA) of the root-inducing (Ri) plasmid into the genome of the plant. The T-DNA carries a set of genes involved in the induction of hairy roots (*rolA, rolB, rolC, rolD*), and for the biosynthesis of auxins, and opines (*iaaM, iaaH, mas1′, mas2′*, and *ags*) [18]. Hairy root genetic transformation has been employed for studying the function of genes [19, 20, 21] or for producing secondary metabolites [22, 23, 24, 25]. This approach provides a major advantage over other methods because of its rapidity and technical simplicity. Moreover, hairy roots are morphologically similar to trichomes [40], making them a suitable system to study candidate genes which are normally functional in similar structures, like trichomes or lupulin glands, respectively.

The present study describes the induction of hairy roots in hop (*H. lupulus* L. cv. Tettnanger) through *A. rhizogenes*-mediated transformation and a method for screening and molecular analysis of putative transgenic hairy roots.

## 2 Materials and methods

### 2.1 Plant material

In vitro hop plants (*H. lupulus* L. cv. Tettnanger) were cultured in 500 ml polystyrene vessels with 20 ml of elongation medium under a 16 h light photoperiod at 21 ± 1 °C according to *Horlemann* et al. [15]. Leaf segments, internodes, petioles and shoots with two nodes from *in vitro* grown plants were used as explants for genetic transformation.

### 2.2 Bacterial strains and binary plasmids

The following *Agrobacterium* strains were used: *A. rhizogenes* K599 (pRi 2659) [26] harboring the binary plasmid pCAMGUSGFP [the T-DNA contained the reporter genes uidA (which codes for b-glucuronidase) and gfp (which codes for green fluorescent protein) under the control of the cauliflower mosaic virus (CAMV) 35S promoter], *A. rhizogenes* 15834 (pRi 15834) [27] carrying the plasmid pCAMGFP-CvMV-GWox-Stisy [the T-DNA contained the reporter gene *gfp* under the control of the cassava vein mosaic virus (CvMV) promoter], and *A. rhizogenes* 15834 (pRi 15834) containing the plasmid pCAMBIA 1303 [the T-DNA contained the reporter genes *mgfp5*, and *uidA*, and the selectable marker gene *hptII* (which codes for hygromycin phosphotransferase) under the control of the CAMV 35S promoter]. The plasmids were introduced into the agrobacteria by the freeze- thaw method [28]. Their presence was corroborated by restriction analysis, as well as by PCR (Polymerase chain reaction) using specific primers for the gene *rolC*. Prior to transformation experiments, a single agrobacteria colony was grown overnight in liquid YEB medium supplemented with $MgSO_4$ (2 mM), rifampicin (50 mg/l) and kanamycin (100 mg/l) on an orbital shaker with 250 rpm at 26 °C. The bacterial suspensions were centrifuged at 3500 rpm for 15 min and then the pellets were resuspended in MS (Murashige and Skoog) medium supplemented with 10 mM MES to an $OD_{600}$ nm between 0.5 and 1.0.

### 2.3 Transformation procedure

In a first experiment, internodes, leaf segments, and double node shoots were co-cultured for 30 min with a solution of *A. rhizogenes* K599::pCAMGUSGFP. Afterwards, the explants were cultivated on co-culture medium (MS-B5 basal medium + 1.43 µM IAA + 9.08 µM TDZ + 2 % sucrose + 0.7 % agar) for 2 days. Then, the explants were collected, washed with distilled water, and cultivated on co-culture medium supplemented with 250 $mgl^{-1}$ cefotaxime [15]. Different co-culture media composition (with or without TDZ and IAA), light conditions (light, and darkness), and co-cultivation time (0, 2, 5, 7, 9, and 11 days) were evaluated.

In a second experiment, leaf segments, internodes, and petioles were co-cultivated with *A. rhizogenes* 15834::pCAMGFP-CvMV-GWox-Stisy following the protocol described by Horlemann et al. [15] with some modifications. Briefly, *Agrobacterium*-infected explants were cultivated on hormone-free co-culture medium supplemented with 250 $mgl^{-1}$ cefotaxime.

In a third experiment, leaf segments and internodes were transformed with *A. rhizogenes* 15834::pCAMBIA 1303 following the protocol described above. In a fourth experiment, the effect of the pre-culture of leaf segments prior to the transformation with *A. rhizogenes* 15834::pCAMBIA 1303 was evaluated. For this purpose, 500 leaf segments were cut two days prior to transformation and cultivated on hormone-free co-culture medium. Moreover, another 500 leaf segments, without pre-culture, were inoculated with the agrobacteria following the protocol described above. In all experiments, explants which had not been exposed to *A. rhizogenes* were included as controls.

### 2.4 Histochemical GUS assays

GUS assays were performed by immersing tissues in the staining buffer [50mM sodium phosphate (pH 7.0), 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 10 mM EDTA, 0,1 %

Triton, 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronide)] for 24 h at 37 °C in the dark [29]. The GUS stained tissue was observed under 10-fold magnification using a stereomicroscope (Stemi SV6, Zeiss, Germany) equipped with a digital camera (EOS 450D, Canon, Japan).

## 2.5 DNA isolation and PCR analysis

Genomic DNA from putative transgenic hairy roots was extracted using the CTAB method [30]. The PCR was performed in a mixture (25 µl) containing 1X Mango Taq PCR buffer, 0.25 mM of each dNTPs, 0.2 µM of each primer, 1.5 mM of $MgCl_2$, 0.5 U Mango Taq$^{TM}$ polymerase (Bioline, Germany) and 1 µl DNA. Three different specific primers were used: *mgfp5* [gfp-f: (5'-atgagtaaaggagaagaacttttcactgg-3') and gfp-r: (5'-ttatttgtatagttcatccatgccatgtg-3'); expected fragment length: 790 bp], *rolC* [rolC-f: (5'-tgtgacaagcagcgatgagc-3') and rolC-r: (5'-gattgcaaacttgcactcgc-3'); expected fragment length: 487 bp], and virG [virG-1f: (5'-ttatctgagtgaagtcgtctcagg-3') and *virG*-1r: (5'-cgtcgcctgagattaagtgtc-3'); expected fragment length: 900 bp]. Primers for *rolC* (X03433.1) and *gfp* (U87624.1) were designed based on sequences available in the GenBank. The primer sequences for *virG* were obtained from *Lee* et al. [41]. The amplification conditions for *mgfp5* and *rolC* were as follows: 94 °C for 3 min followed by 35 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and a final step of 72 °C for 10 min. For *virG* the amplification conditions were as follows: 96 °C for 5 min followed by 36 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final step of 72 °C for 10 min [41]. PCR products were evaluated on 1.5 % (w/v) agarose gel and stained with DNA Stain G (Serva, Germany). The plasmid pCAMBIA 1303 was used as positive control to test the presence of the genes *mgfp5* and *rolC*, whereas DNA isolated from *A. rhizogenes* was used as positive control for *virG*.

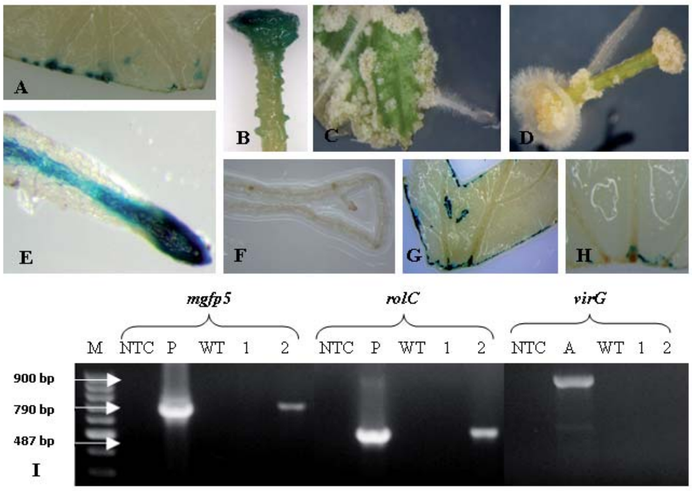

**Fig. 1** ***Agrobacterium rhizogenes*-mediated transformation of *Humulus lupulus* L. cv. Tettnanger. Transient *uidA* expression in (A) leaf segments and (B) internodes. Hairy roots induction in (C) leaf segments and (D) internodes. (E) *uidA* expression in transgenic roots. (F) Wildtype root showing no *uidA* activity. Transient *uidA* expression in leaf segments without pre-culture (G) and with pre-culture (H), (I): PCR analysis showing the presence of the genes *mgfp5* and *rolC* and absence of the gene *virG* in transgenic hop hairy roots. M: molecular weight marker (100 bp), NTC: negative control (PCR reaction mix without template), P: positive control (pCAMBIA 1303 DNA), WT: wildtype roots (non-transformed control) , A: *A. rhizogenes* 15834 DNA, 1-2: putative transgenic hairy root**

## 3 Results and discussion

*A. rhizogenes* strain K599 (pRi 2659) and *A. rhizogenes* strain 15834 (pRi 15834) were tested for their ability to induce the formation of hairy roots on hop explants. Using *A. rhizogenes* strain K599 with different organ types, co-culture media composition, light conditions, and co-cultivation times, no hairy root formation was observed in any of the treatments (data not shown). It has been reported that *A. rhizogenes* strains, which differ in their virulence, determines the successful induction of hairy roots [20, 31, 32, 33]. *A. rhizogenes* K599 (pRi 2659) is a low virulence strain and has been used for genetic transformation of *Glycine max*, *Lotus corniculatus*, *Phaseolus vulgaris*, and *Cucumis melo* [34, 35, 36, 37].

As an alternative *A. rhizogenes* strain 15834 (pRi 15834) was used for the transformation of hop explants. The different response of leaf segments, internodes, and petioles after inoculation with *A. rhizogenes* 15834::pCAMGFP-CvMV-GWox-Stisy is shown in table 1. The highest percentage of explants with roots and total number of roots were obtained when infected leaf segments were cultivated with the adaxial surface in contact with the co-culture medium (Table 1). No hairy root formation was observed in non-transformed controls. Furthermore, this confirmed previous reports that the type of inoculated organ is an important factor affecting *A. rhizogenes*-mediated transformation [20, 21, 24, 33]. Only *A. rhizogenes* strain 15834 was capable of inducing hairy root formation. This difference could be due to genotypic differences as well as different Ri plasmid they were harboring. Plasmid pCAMGFP-CvMV-GWox-Stisy contains the reporter gene *gfp*, which has been used for screening of transgenic roots in *Daucus carota*, *Capsicum annuum*, *Saponaria vaccaria*, and *Cucumis melo* [24, 32, 37, 38]. However, in *Humulus lupulus* the fluorescence of *gfp* cannot be used to unambiguously differentiate transgenic from non-transgenic roots (data not shown). This could be attributed to hop secondary metabolites fluorescing at a similar wavelength as *gfp* [39].

After the conditions for the transformation were optimized, in a third experiment, leaf segments and internodes were transformed with *A. rhizogenes* 15834 harboring the plasmid pCAMBIA 1303, which contains the reporter genes *uidA* and *mgfp5*. Twelve days after co-culture with the agrobacteria, leaf segments (Fig. 1A) and internodes (Fig. 1B) showed blue staining from the enzymatic activity of *b*-glucuronidase. Non-transformed controls (leaf segments and internodes) were not stained under similar reaction conditions (data not shown). Previously, hop transformation had been carried out using *uidA* as reporter gene [15]. Sixteen days after inoculation, of 207 inoculated leaf segments 3 % yielded hairy roots (Fig. 1C). In this case, a GUS assay was not performed because of the insufficient quantity of roots. On the other hand, of 219 inoculated internodes, 35 % yielded hairy roots 12 days after inoculation (Fig. 1D), of which 5 % (3 of 60) showed blue staining. The *uidA* expression was observed in the root tip and central cylinder of the putative transgenic hairy roots (Fig. 1E), whereas non-transformed controls did not exhibit *uidA* activity (Fig. 1F). Root induction was not observed in non-transformed controls (data not shown).

Regarding the effect of the pre-culture of explants prior to the transformation with *A. rhizogenes* 15834::pCAMBIA 1303, it was demonstrated that leaf segments without pre-culture (Fig. 1G) showed a stronger *uidA* expression than leaf segments with pre-culture (Fig. 1H). Moreover, of 500 leaf segments without pre-culture, 23 % yielded hairy roots 27 days after inoculation. On the other hand, of 500 leaf segments pre-cultured on hormone-free co-culture medium prior to the transformation, 11 % yielded hairy roots 27 days after inoculation.

Putative transgenic hairy roots (8-weeks-old) were pooled and tested by PCR for the presence of the T-DNA genes transferred from the binary vector (*mgfp5*), and the Ri plasmid (*rolC*), as well as for false positives caused by *A. rhizogenes* contamination (indicated by the presence of *virG* gene). The specific *mgfp5* (790 bp) and *rolC* (487 bp) fragments were amplified in 1 of 2 pooled putative transgenic hairy roots (Fig. 1I). No DNA fragments were amplified by PCR in wildtype plants (Fig. 1I). Moreover, the *virG* gene was not detected in transgenic hairy roots, indicating no contamination with *A. rhizogenes* (Fig. 1I).

## 4 Conclusions

According to the best of our knowledge this is the first report for induction of hairy roots in hop (*Humulus lupulus* L. cv. Tettnanger). Instead of the 6-9 months-time required for regenerating hop transgenic plants following *A. tumefaciens* transformation [11,15,16], in the present study, production of hairy roots takes only 4 weeks. Therefore, prior to producing hop transgenic plants using *A. tumefaciens*, transgenic hairy roots provide a fast and easy tool for screening and evaluating a larger number of candidate genes, for instance, from the flavonoid and/or phloroglucinol biosynthetic pathways.

In order to improve the transformation efficiency different strategies including sonication assisted transformation (SAAT) and co-cultivation with two *Agrobacterium* strains will be evaluated. Moreover, a liquid culture system for root growth will be established.

## 5 Acknowledgements

The support of Hop Growers Cooperative (Tettnang, Germany) is gratefully acknowledged. The scholarship from German Academic Exchange Service (DAAD) and partial support from the University of Costa Rica are gratefully acknowledged by A. Gatica-Arias. The authors thanked Griselda Arrieta (Centro de Investigación en Biología Celular y Molecular, University of Costa Rica) for providing the pCAMBIA 1303 plasmid.

## 6 References

1. De Keukeleire, J.; Ooms, G.; Heyerick, A.; Roldan-Ruiz, I.; Van Bockstaele, E. and De Keukeleire D.: Formation and accumulation of α-acids, β-acids, desmethylxanthohumol, xanthohumol during flowering of hops (*Humulus lupulus* L.), Journal of Agricultural Food and Chemistry, **51** (2003), no. 15, pp. 4436 4441.
2. Zanoli P. and Zavatti M.: Pharmacognostic and pharmacological profile of *Humulus lupulus* L., Journal of Enthnopharmacology, **116** (2008), no. 3, pp. 383 - 396.
3. Tagashira, M.; Watanabe, M. and Uemitsu, N.: Antioxidative activity of hop bitter acids and their analogues, Bioscience Biotechology and Biochemistry, **59** (1995), no. 4, pp. 740 - 742.
4. Miranda, C.; Yang, Y.; Henderson, M.; Stevens, J.; Santana-Rios, G. and Deinzer, M.: Prenylflavonoids from hops inhibit the metabolic activation of the carcinogenic heterocyclic amine 2-amino-3-methylimidazol [4, 5-f] quinoline, mediated by cDNA-expressed human CYP1A2, Drug Metabolism and Disposition., **28** (2000), no. 11, pp. 1297 - 1302.
5. Milligan, S.; Kalita, J.; Pocock, V.; Heyerick, A.; De Cooman, L. and Rong, H.: Oestrogenic activity of the hop phyto-oestrogen, 8-prenylnaringenin, Reproduction, **123** (2002), no. 2, pp. 235 - 242.
6. Chen, W. and Lin, J.: Mechanisms of cancer chemoprevention by hop bitter acids (beer aroma) through induction of apoptosis mediated by Fas and Caspase Cascades, Journal of Agricultural Food and Chemistry, **52** (2004), no. 1, pp. 55 - 64.
7. Gerhäuser, C.: Beer constituents as potential cancer chemopreventive agents, European Journal of Cancer, **41** (2005), no. 13, pp. 1941 - 1954.
8. Lamy, V.; Roussi, S.; Chaabi, M.; Gossé, F.; Schall, N.; Lobstein, A. and Raul F.: Chemopreventive effects of lupulone, a hop β-acid, on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis, Carcinogenesis, **28** (2007), no. 7, pp. 1575 - 1581.
9. Dixon, R.A. and Steele, C.L.: Flavonoids and isoflavonoids – a gold mine for metabolic engineering, Trends Plant. Sci., **4** (1999), pp. 394 - 400.
10. Capell, T.; Christou, P.: Progress in plant metabolic engineering, Current Opinion in Biotechnology, **15** (2004), no. 2, pp. 148 - 154.
11. Gatica-Arias, A.; Farag, M.A.; Stanke, M.; Matoušek, J.; Wessjohann, L. and Weber, G.: Flavonoid production in transgenic hop (*Humulus lupulus* L.) altered by PAP1/MYB75 from *Arabidopsis thaliana* L., Plant Cell Reports, **31** (2012), no.1, pp. 111 - 119.
12. Borevitz, J.; Xia, Y.; Blount, J.; Dixon, R. and Lamb, C.: Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis, Plant Cell, **12** (2000), no. 12, pp. 2383 - 2394.
13. Tohge, T.; Nishiyama, Y.; Hirai, M.; Yano, M.; Nakajima, J.; Awazuhara, M.; Inoue, E.; Takahashi, H.; Goodenowe, D.; Kitayama, M.; Noji, M.; Yamazaki, M. and Saito, K.: Functional genomics by integrated analysis of metabolome and transcriptome of Arabidopsis plants overexpressing an MYB transcription factor, Plant Journal, **42** (2005), no. 2, pp. 218 - 235.
14. Matoušek, J.; Vrba, L.; Škopek, J.; Orctová, L.; Pešina, K.; Heyerick, A.; Baulcombe, D. and De Keukeleire, D.: Sequence analysis of a "true" chalcone synthase (chs_H1) oligofamily from hop (*Humulus lupulus* L.) and PAP1 activation of chs_H1 in heterologous systems, Journal of Agricultural and Food Chemistry, **54** (2006), no. 20, pp. 7606 - 7615.
15. Horlemann, C.; Schwekendiek, A.; Höhnle, M. and Weber, G.: Regeneration and *Agrobacterium*-mediated transformation of hop (*Humulus lupulus* L.), Plant Cell Reports, **22** (2003), no. 3, pp. 210 - 217.
16. Schwekendiek, A.; Spring, O.; Heyerick, A.; Pickel, B.; Pitsch, N.; Peschke, F.; De Keukeleire, D. and Weber, G.: Constitutive expression of a grapevine stilbene synthase gene in transgenic hop (*Humulus lupulus* L.) yields resveratrol and its derivatives in substantial quantities, Journal of Agricultural Food and Chemistry, **55** (2007), no. 17, pp.7002 - 7009.
17. Batista, D.; Fonseca, S.; Serrazina, S.; Figueiredo, A. and Salomé Pais, M.: Efficient and stable transformation of hop (*Humulus lupulus* L.) var. Eroica by particle bombardment, Plant Cell Reports, **27** (2008), no. 7, pp. 1185 - 1196.

18. Georgiev, M.I; Pavlov, A.I. and Bley T.: Hairy root type plant in vitro system as sources of bioactive substances, Applied Microbiology and Biotechnology, **74** (2007), no. 6, pp. 1175 - 1185.
19. Bosselut, N.; Van Ghelder C.; Claverie M.; Voisin R.; Onesto J.P.; Rosso M.N.; Esmenjaud D.: *Agrobacterium rhizogenes*-mediated transformation of Prunus as an alternative for gene functional analysis in hairy-roots and composite plants, Plant Cell Reports, **30** (2011), no. 7, pp. 1313 - 1326.
20. Alpizar, E.; Dechamp, E.; Espeout, S.; Royer, M.; Lecouls, A.C.; Nicole, M.; Bertrand, B.; Lashermes, P. and Etienne, H.: Efficient production of *Agrobacterium rhizogenes*-transformed roots and composite plants for studying gene expression in coffee roots, Plant Cell Reports, **25** (2006), no. 9, pp. 959 - 967.
21. Kifle, S.; Shao, M.; Jung, C. and Cai D.: An improved transformation protocol for studying gene expression in hairy roots of sugar beet (Beta vulgaris L.), Plant Cell Reports **18** (1999), no. 6, pp. 514 - 519.
22. Kim, J.A.; Kim Y.S. and Choi, Y.E.: Triterpenoid production and phenotypic changes in hairy roots of *Codonopsis lanceolata* and the plants regenerated from them, Plant Biotechnology Reports, **5** (2011), no. 3, pp. 255 - 263.
23. Zaraté, R.: Tropane alkaloid production by *Agrobacterium rhizogenes* transformed hairy root cultures of *Atropa baetica* Willk. (Solanaceae), Plant Cell Reports, **18** (1999), no. 5, pp. 418 - 423.
24. Aarrouf, J.; Castro-Quezada, P.; Mallard, S.; Caromel, B.; Lizzi, Y. and Lefebvre, V.: Agrobacterium rhizogenes-dependent production of transformed roots from foliar explants of pepper (*Capsicum annuum*): a new and efficient tool for functional analysis of genes, Plant Cell Reports, **31** (2012), no. 2, pp. 391 - 401.
25. Georgiev, M.I.; Ludwig-Müller J.; Alipieva K.; Lippert A.: Sonication-assisted *Agrobacterium rhizogenes*-mediated transformation of *Verbascum xanthophoeniceum* Griseb. for bioactive metabolite accumulation, Plant Cell Reports, **30** (2011), no. 5, pp. 859 - 866.
26. Savka, M.A.; Ravillion, B.; Noel, G.R. and Farrand, S.K.: Induction of hairy roots on cultivated soybean genotypes and their use to propagate the soybean cyst nematode, Phytopathology, **80** (1990), no. 5, pp. 503 - 508.
27. Lippincott, J.A.; Beiderbeck, R. and Lippincott, B.B.: Utilization of octopine and nopaline by Agrobacterium, Journal of Bacteriology, **116** (1973), no. 1, pp. 378 - 383.
28. Sambrook, J.; Fritsch, E.F. and Maniatis, T.: Molecular cloning: a laboratory edition, 2nd ed.; Cold Spring Harbour Laboratory Press, New York, 1989.
29. Jefferson, R.A.: Assaying chimeric genes in plants: the GUS gene fusion system, Plant Molecular Biology Reporter, **5** (1987), no. 4, pp. 387 - 405.
30. Fleischer, R.; Horlemann, C.; Schwekendiek, A.; Kling, C. and Weber, G.: AFLP fingerprinting in hop: analysis of the genetic variability of the Tettnang variety, Genetic Resources and Crop Evolution, **51** (2004), no. 2, pp. 211 - 220.
31. Akramian, M.; Tabatabaei S.M.F. and Mirmasoumi, M.: Virulence of different strains of *Agrobacterium rhizogenes* on genetic transformation of four *Hyoscyamus species*, American-Eurasian Journal of Agriculture and Environmental Science, **3** (2008), no. 5, pp. 759 - 763.
32. Baranski, R.; Klocke, E. and Schumann, G.: Green fluorescent protein as an efficient selection marker for *Agrobacterium rhizogenes* mediated carrot transformation, Plant Cell Reports, **25** (2006), no. 3, pp. 190 - 197.
33. Kajikawa, M.; Morikawa, K.; Abe, Y.; Yokota, A. and Akashi, K.: Establishment of a transgenic hairy root system in wild and domesticated watermelon (*Citrullus lanatus*) for studying root vigor under drought, Plant Cell Reports, **29** (2010), no. 7, pp.771 - 8.
34. Mankin, S.L.; Hill, D.S.; Olhoft, P.M.; Toren, E.; Wenck, A.R.; Nea, L.; Xing, L.; Brown, J.A.; Fu, H.; Ireland, L.; Jia, H.; Hillebrand, H.; Jones, T. and Song, H.S.: Disarming and sequencing of *Agrobacterium rhizogenes* strain K599 (NCPPB2659) plasmid pRi2659, In Vitro Cellular Developmental Biology-Plant, **43** (2007), no. 6, pp. 521 - 535.
35. Jian, B.; Hou W.; Wu C.; Liu, B.; Liu, W.; Song, S.; Bi, Y. and Han, T.: *Agrobacterium rhizogenes*-mediated transformation of Superroot-derived *Lotus corniculatus* plants: a valuable tool for functional genomics, BMC Plant Biology, **9** (2009), pp. 78.
36. Estrada-Navarrete, G.; Alvarado-Affantranger, X.; Olivares, J.E.; Guillén, G.; Díaz-Camino, C.; Campos, F.; Quinto, C.; Gresshoff, P.M.; Sanchez, F.: Fast, efficient and reproducible genetic transformation of *Phaseolus* spp. by *Agrobacterium rhizogenes*, Nature Protocols, **2** (2007), pp. 1819 - 1824.
37. Pak, H.K.; Sim, J.S.; Rhee, Y.; Ko, H.R.; Ha, S.H.; Yoon, M.S.; Kang, C.H.; Lee, S.; Kim, Y.H. and Hahn, B.S.: Hairy root induction in Oriental melon (*Cucumis melo*) by *Agrobacterium rhizogenes* and reproduction of the root-knot nematode (Meloidogyne incognita), Plant Cell Tissue and Organ Culture, **98** (2009), no. 2, pp. 219 - 228.
38. Schmidt, J.F.; Moore, M.D.; Pelcher, L.E.; and Covello, P.S.: High efficiency *Agrobacterium rhizogenes*-mediated transformation of *Saponaria vaccaria* L. (Caryophyllaceae) using fluorescence selection, Plant Cell Reports, **26** (2007), no. 9, pp.1547 - 1554.
39. Lin, M.H.; Gresshoff1, P.M.; Indrasumunar, A. and Ferguson, B.J.: pHairyRed: A novel binary vector containing the DsRed2 reporter gene for visual selection of transgenic hairy roots, Molecular Plant, **4** (2011), no. 3, pp. 537 - 545.
40. Wagner, G.J.: Secreting glandular trichomes: more than just hairs, Plant Physiology, **96** (1991), no. 3, pp. 675 - 679.
41. Lee, M.H.; Yoon, E.S.; Jeong, J.H. and Choi, Y.E.: *Agrobacterium rhizogenes*-mediated transformation of *Taraxacum platycarpum* and changes of morphological characters, Plant Cell Reports, **22** (2004), no. 11, pp. 822 - 827.

*Received 1 June, 2012, accepted 25 July, 2012*