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RESEARCH ARTICLE

A reliable and efficient protocol for inducing genetically transformed roots in medicinal plant *Nepeta pogonosperma*

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Abstract *Nepeta pogonosperma* is an important medicinal plant with anti-inflammatory effects. An efficient and reliable transformation system for this plant was developed through optimization of several factors which affected the rate of *Agrobacterium rhizogenes* mediated transformation. Five bacterial strains, A4, ATCC15834, LBA9402, MSU440 and A13, two explant types, leaves and stems, and several co-cultivation media were examined. The maximum rate of hairy root induction was obtained from stem explants using MSU440 and ATCC15834 bacterial strains. A drastic increase in the frequency of transformation (91 %) was observed when MS medium lacking NH_4NO_3, KH_2PO_4, KNO_3 and $CaCl_2$. Hairy root lines were confirmed by polymerase chain reaction (PCR) using primers of the *rol*B gene. According to Southern blot analysis, one T-DNA copy was inserted into each of the hairy root lines. In the present study, transgenic hairy roots have been obtained trough genetic transformation by *A. rhizogenes* harbouring two plasmids, the Ri plasmid and pBI121 binary vector harbouring *gus* reporter gene. Expression of the *gus* gene in transgenic hairy root was confirmed by histochemical GUS assay.

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Introduction

Medicinal plants are the most important source of drugs for people all around the world (Tripathi and Tripathi 2005). The global demand for herbal medicine is growing (Khan et al. 2009). *Nepeta* genus has 67 species growing in different regions of Iran; most of them are used in traditional medicine for their anticonvulsant, anti-cough and asthma, antiseptic and diuretic effects. *Nepeta pogonosperma* Jamzad et Assadi was identified as a new species in 1984 (Jamzad and Assadi 1984). Sefidkon and Akbarinia (2003) has demonstrated that 4aα-7α-7aβ-nepetalactone and 1, 8-cineole are the main compounds in *N. pogonosperma* essential oil. Anti-inflammatory effects of these compounds have also been investigated in previous studies (Silva et al. 2003; Miceli et al. 2005).

Biotechnological tools such as in vitro regeneration and genetic transformation are important for multiplication and metabolic engineering of medicinal plants. *Agrobacterium rhizogenes* is a gram-negative soil bacterium responsible for hairy root induction at the site of infection by transferring T-DNA of Ri plasmid. Hairy root grows much faster and it maintains genetic and biochemical stability. Hairy root can be cultivated in a simple medium without phytohormones (Tao and Li 2006; Pratapchandran and Potty 2011). Moreover, hairy root culture are becoming attractive for expressing recombinant proteins (Ghiasi et al. 2012; Ono and Tian 2011).

Several factors affect the rate of *A. rhizogenes* mediated transformation. Henzi et al. (2000) indicated that the use of 200 mM acetocyringone in LB medium for bacterial growth, 50 mM acetocyringon and 1 mM arginine in the co-cultivation medium showed significant improvement in *A. rhizogenes* transformation of *Brassica oleracea* L. In another study,

Sharafi et al. (2013d) modified the medium of co-cultivation (lacking KH_2PO_4, NH_4NO_3, KNO_3 and $CaCl_2$) for reaching to high efficient transformation of *Papaver bracteatum*.

In this study, we describe an efficient protocol for the development of hairy root culture as a biotechnological tool for secondary metabolite production from *N. pogonosperma*. The improved transformation was achieved by modifying macroelement compounds in co-cultivation medium. Different strains of *A. rhizogenes* and *N. pogonosperma* explants were evaluated in this study. Also transgenic hairy roots have been obtained via transformation by *A. rhizogenes* harbouring pBI121 binary vector harbouring *gus* reporter gene. To the best of our knowledge, it is the first study on *N. pogonosperma* transformation and hairy root production.

Materials and methods

Seed sterilization and germination

Seeds of *N. pogonosperma* were procured from Alamout Medicinal Plant Research Center (Qazvin province, Iran). The seeds were surface sterilized. They were cultured in half strength MS medium (Murashige and Skoog 1962), solidified with 0.7 % (w/v) agar. The medium was adjusted to pH 5.8 before adding agar. The cultures were maintained under a 16 h photoperiod regime with fluorescent light at 25° C in a culture room.

Preparation of A. rhizogenes strains

Five strains of *A. rhizogenes* (ATCC 15,834, LBA9402, A4, A13, and MSU440) (All of them were provided by the bank of microbes at the National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran) were used in this study. A single bacterial colony from each strain was inoculated in 10 ml of LB medium. The culture was placed on rotary shaker (160 rpm) at 28 °C for 24 h. The bacterial suspensions were centrifuged at 4,000 rpm for 10 min at 4 °C. The pellets were re-suspended in 20 ml MS liquid medium supplemented with 100 μM acetocyringone.

Co-cultivation and establishment of hairy root culture

Explants (leaf and stem) were pre-cultured for two days on MS medium supplemented with 2 mg/l 6-benzyl-aminopurine (BA). The explants were immersed in bacterial suspension (optical density at 600 nm, OD = 0.5-0.8) for 10 min and then blotted on sterile filter paper and incubated in a co-cultivation medium consisted of MS salts and vitamins along with 50 mg/l sucrose and 100 μM acetocyringone (designated as Medium 1). This medium was subsequently adjusted by removing the following compounds: KH_2PO_4 (Medium 2); KH_2PO_4, $CaCl_2$ (Medium 3); KH_2PO_4, NH_4NO_3, KNO_3 (Medium 4); KH_2PO_4, NH_4NO3, KNO_3, $CaCl_2$ (Medium 5) based on our previous studies (Sharafi et al. 2013a,b,c). Control explants were used with similar treatments without inoculation with *A. rhizogenes*. After two days, they were transferred to MS medium containing 400 mg/l cefotaxime to eliminate *A. rhizogenes*. They were sub-cultured every week by reducing the concentration of cefotaxime.

Transgenic hairy roots induction using pBI121 binary vector

The best bacterial strain and explant (MSU440 and stem, respectively) obtained from the previous experiment were selected for this part of study. The pBI121 vector was mobilized by freeze–thaw method into *A. rhizogenes* strain MSU440. Bacteria were grown in liquid LB medium supplemented with 50 mg/l kanamycin to mid-growth phase (OD_{600} =0.6). The cells were collected by centrifugation and resuspended at a density of OD_{600} = 0.8 in liquid inoculation medium (MS salts-NH_4NO_3, KNO_3, KH_2PO_4, and vitamins containing 50 g sucrose/l and 100 μM acetocyringone).

Polymerase chain reaction analysis and Southern hybridization

Genomic DNA was extracted from 8 unrelated samples of hairy root and a normal root (control) of *N. pogonosperma* using a modified CTAB protocol for DNA extraction from medicinal plant species (Ibrahim, 2011). PCR analysis was done using the *rol*B specific primers for samples. Primer sequences used are as follows: 5′-GCTCTTGCAGTGCTAG ATTT-3′ and 5′-GAAGGTGCAAGCTACCTCTC-3′. Amplification products were separated by electrophoresis on 0.8 % agarose gel in 0.5× TBE buffer, stained with ethidium bromide and visualized under UV trans-illuminator.

Genomic DNA (15 μg) of hairy roots and control (normal root) of *N. pogonosperma* were digested using *Xba*I (Fermentas Co., Germany) separated on 0.8 % agarose gel and subsequently transferred to a nylon membrane. Probe was designed on the basis of *rol*B sequence, using the DIG DNA labeling Kit (Roche Co., Germany). Prehybridization, hybridization, washing and detection were performed according to the instruction manual of the DIG Labeling and Detection System (Roche Co., Germany). Also, PCR was performed using genomic DNA of transgenic hairy roots as a target and the oligonucleotide primers for *gus* gene as follow: Forward primer: 5′-GGTGGTCAGTCCCTTATGTTACG-3′ and reverse primer 5′- CCGGCATAGTTAAAGAAATCATG -3′.

Histochemical GUS assay

The hairy roots and roots from a normal plant were examined for histochemical GUS expression by soaking in X-Gluc solution (Jefferson 1987).

Shoot regeneration from hairy roots

Hairy roots and tumors induced by *A. rhizogenes* were transferred to regeneration media. MS solid media supplemented 0.5 mg/l BA in companion with 0.1 mg/l NAA, pH 5.8 was used as regeneration medium. After one month, regenerated shoots were excised and cultivated on root induction medium (MS medium supplemented with 0.5 mg/l IBA).

Statistical analysis

The experiments were laid on a completely randomized design (CRD) with 3 replications and 9 explants cultured in each Petri dish. The data collected were subjected to analysis of variance test. The means were compared using Duncan's multiple range tests.

Results

Effect of *A. rhizogenes* strains, types of explant and different co-cultivation medium

Five different *Agrobacterium rhizogenes* strains: ATCC15834, A4, LBA9402, A13, MSU440 were examined for transformation ability. Hairy roots of *Nepeta pogonosperma* were initiated from stem and leaf explants after one week. They were transferred to 1/2 MS liquid medium for more growth after three weeks (Fig. 1). *N. pogonosperma* stems and leaves were susceptible to infection by all *A. rhizogenes* strains, as shown by the percentage of each explant from which hairy roots emerged. The highest rate of hairy root induction was found using strain MSU440 followed by strains A13 and ATCC15834, respectively. All of the *A. rhizogenes* strains led to hairy root induction and also caused the formation of tumorigenic calli using the stem explants. Stem explants were the best explants for *A. rhizogenes* mediated transformation in *N. pogonosperma*. Leaf explants showed necrosis with a low rate of hairy root induction (data not shown).

Stem explants as a suitable explant were evaluated for testing the effect of elimination of macro element salts in co-cultivation media with five *A. rhizogenes* strains. The maximum rate of transformation in full strength MS medium (medium 1), was observed at 61 % in the ATCC15834 strain. On the other hand, using medium 3 or 4 showed 91 and 87.6 % hairy root induction by the same strain.

Transformation frequency in medium 5, was significantly increased in comparison with the full strength MS medium (medium 1) in all strains and resulted in 50.6, 50.6, 69.3, 88.6 and 81.3 % hairy root induction frequency in A4, LBA9402, A13, MSU440, and ATCC15834 strains, respectively (Fig. 2).

PCR and southern bolt analysis

PCR was performed to investigate presence of the *rol*B gene transferred from Ri plasmid. PCR led to amplification of *rolB* gene in all transgenic hairy root lines. T-DNA copy number in

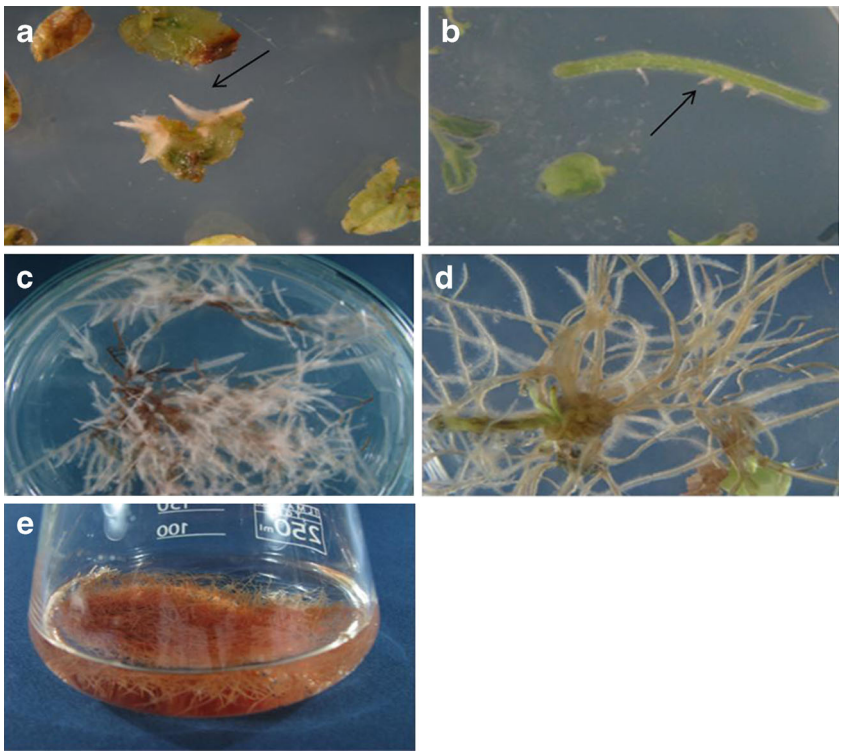

Fig. 1 *A. rhizogenes* mediated transformation in *N. pogonesperma;* **a** hairy root induction in leaf explants after 3 weeks of inoculation; **b** induction of hairy root on wound site of stem, after 4 weeks of inoculation using strain MSU440; **d** Hairy root growth after 4 weeks of inoculation using ATCC15834 strain; **c** Hairy root growth after 4 weeks of inoculation using strain MSU440; **e** Hairy root culture in liquid MS medium; **f, g, h** induction of adventitious shoot from hairy roots and calli; **i** growth of regenerated shoots after one month

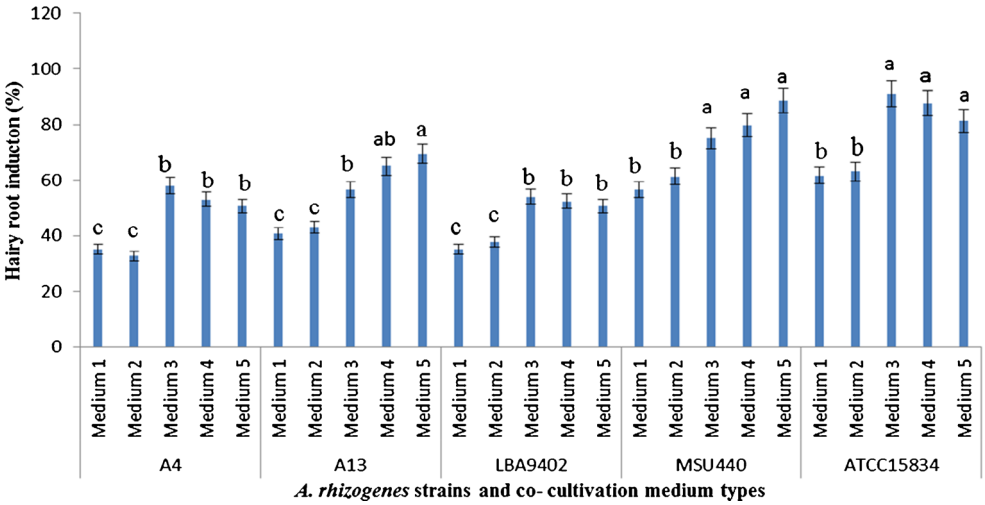

Fig. 2 Effect of different *A. rhizogenes* strains and co-cultivation media on percentage of hairy root induction in *N. pogonesperma.* The data were obtained as a mean of four replications. The different letters denote a statistically significant difference at $P \leq 0.05$, as determined by Duncan's multiple range test. Vertical lines represent standard errors

transgenic hairy root lines was determined by Southern blot analysis on nylon membranes and the results showed that one T-DNA copy number was inserted into all hairy root lines, while no hybridization signal was observed in the untransformed root (Fig. 3).

Transgenic hairy root production

Twenty nine independent hairy root lines were separated from 22 explants (total explants were 24) and culture in MS liquid media 3 weaks after inoculation. Approximately, 35 % (10 lines) of the hairy root lines were transgenic as analysed by PCR and histochemical GUS assay (Fig. 4 and Fig. 5). Transformation efficiency (the frequency of co-cultivated leaf explants which produced independent hairy-root transgenic lines) of 41.7 % was obtained.

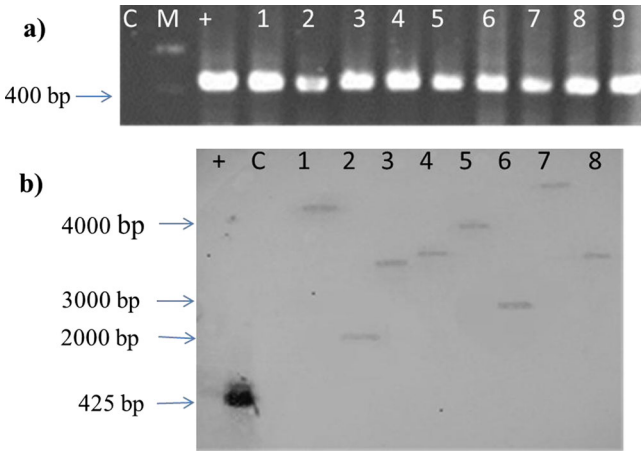

Fig. 3 Molecular analysis of hairy roots. a) PCR analysis for detection of the *rolB* gene in hairy roots of *N. pogonesperma* M: Molecular size marker (1 kb ladder Fermentase); 1–9: hairy roots, C: negative control (non-transformed root); +: positive control (Ri plasmid). b) Southern blot analysis of hairy roots (1–8) and an untransformed root (C). DNA samples were digested with *Xba*I and hybridized to *rolB* probe. Molecular markers are indicated on the left

Shoot regeneration from hairy root cultures

After 2 weeks most hairy root segments induced callus in regeneration medium. Some small shoot points were formed on the surface of calli from hairy roots after 3 to 4 weeks (Fig. 6 a, b, c). the induced shoots were cut and culture on MS medium for more growth (Fig. 6 d). then they were cultivated on root induction medium for rooting.

Discussion

Previous studies have shown that several factors such as genotype, explant, physical and chemical factors, strain of *A. rhizogenes* can affect genetic transformation by *A. rhizogenes* (Kumar et al. 1991; Sharafi et al. 2013b). Selection of explant type and effective *A. rhizogenes* strain

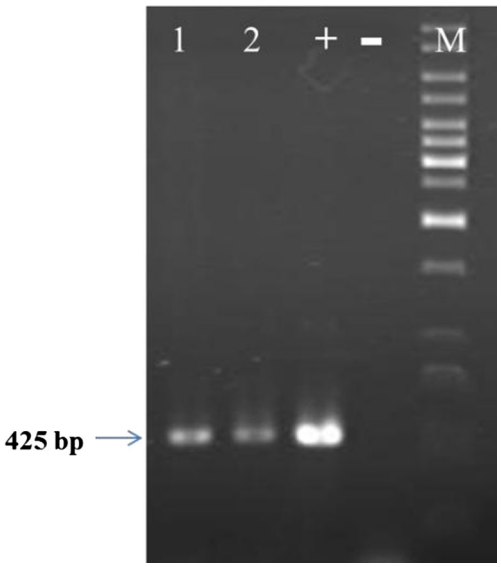

Fig. 4 PCR analysis of hairy roots (1,2) and normal root (−); M: 1 kb size marker; positive control (+) is plasmid DNA (pBI121)

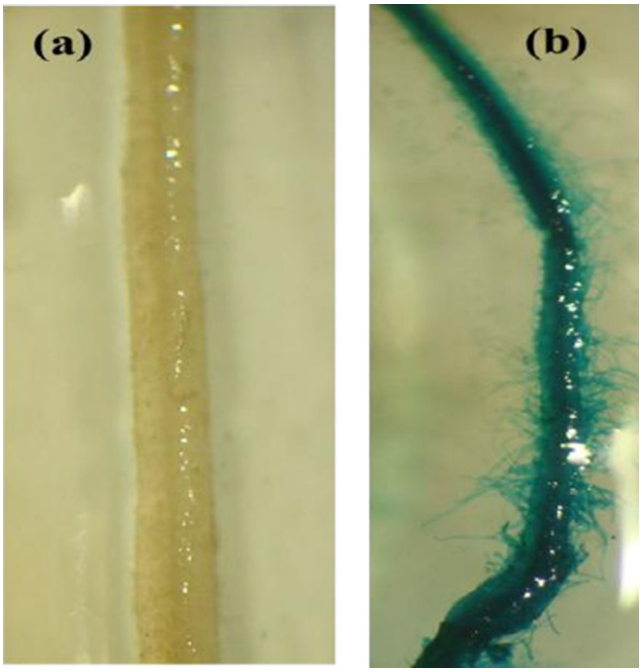

Fig. 5 Histochemical GUS assay; **a** normal root; **b** hairy root

is very important factor affecting frequency of transformation. In present study it was observed that stem explants were much susceptible than leaf to infection by all *A. rhizogenes* strains which were tested. The highest frequency of hairy root induction was achieved in case of MSU440 and ATCC15834 strains using stem explants. Strain A4 produced low hairy root induction in comparison to other strains. This could be due to presence of different plasmids in this strain (Nguyen et al. 1992).

Co-cultivation is very important in transformation processes, since bacterial attachment, T-DNA transfer and integration to plant genome is made at this stage. Transformation can increase according to changes in co-culture media; our results suggest that macro-elements in co-cultivation media have inhibitory effects on the transformation of *N. pogonosperma* via *A. rhizogenes*. Compared to the MS full strength medium, cell proliferation of *A. rhizogenes* around the explants during co-cultivation in media four and five was highly increased. The bacterial overgrowth in these media proved the inhibitory effect of macro-elements on the proliferation of *A. rhizogenes* during co-cultivation time (data not shown). Previous studies showed that low level of PO_4 can activate the expression of *virG* and could be a positive signal to induce the infection of plants (Winans 1990; Dupre et al. 2000) indicating that a medium with lacking mineral components was the best co-cultivation medium to achieve the highest rate of transformation in *Ginkgo biloba*. Similar result was reported in the case hairy root induction of *Papaver bracteatum* (Sharafi et al. 2013c).

The main outcome of the present study is introduction of a reliable and well defined protocol to induce high frequency of

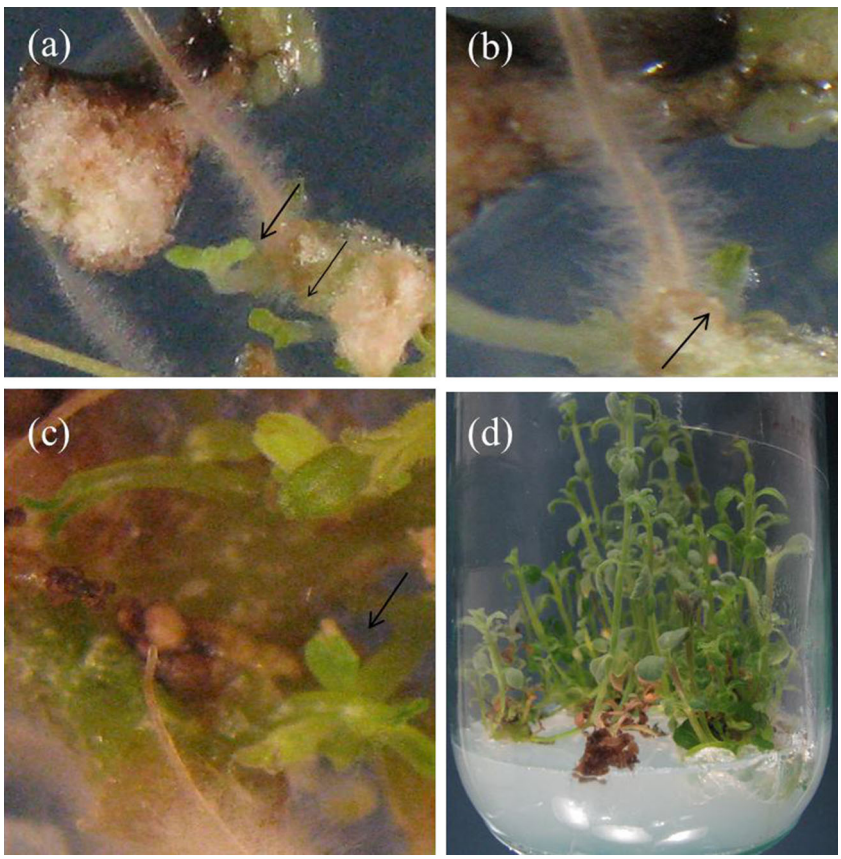

Fig. 6 Adventitious shoot regenerated from hairy roots of *N. pogonesperma;* **a, b** induction of adventitious shoot from hairy root after 3 weeks **c** growth of regenerated shoots after 4 weeks from hairy root; **d** shoots originated from hairy roots after 6 weeks

hairy root induction and increase the growth rate in *N. pogonosperma.* The *A. rhizogenes* strains MSU440 and ATCC15834, stem explant and medium lacking KH_2PO_4, NH_4NO_3, KNO_3, $CaCl_2$ as best strains, explant and co-cultivation medium respectively are proposed as most suitable for *N. pogonosperma* transformation. Transformation efficiency can be over 40 % using binary vector (co-transformation of *rol* gene and target gene) by this protocol. The transgenic hairy roots can be used as explants for plantlet regeneration to obtain stable transgenic plants (Sharafi et al. 2013a).

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