An Improved *Agrobacterium tumefaciens* Mediated Transformation of *Artemisia annua* L. by Using Stem Internodes as Explants

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Abstract

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Transformation of *Artemisia annua*, which produces the sesquiterpenoid endoperoxide artemisinin widely used for the treatment of malaria, has been hampered by the low efficiency of adventitious shoot and root formation on a selective medium containing additional compounds for *Agrobacterium* decontamination. Here we identified several factors which were all shown to be of importance for optimization of *Artemisia annua* transformation. Results indicated that stem internodes showed better resistance capacity to *Agrobacterium* decontaminator than leaves did. *Agrobacterium tumefaciens* with an optical density (OD) value of 0.2–0.5 plus 100 μmol of acetosyringone per litre of solution gave the best transformation efficiency. Moreover, kanamycin at 30 mg/l in the culture medium was effective in suppressing the growth of non-transformed tissue. Furthermore, transgenic shoots required an early induction of rooting. In addition, dimethyl sulphoxide considerably improved the rooting of shoots. The present work provides rapid and reproducible transformation and regeneration of *A. annua*.

Keywords: anti-malaria; artemisinin; stem internode; traditional Chinese medicine; transgene

Artemisia annua L. has been traditionally used to treat fever, inflammation and malaria for a long time in China. And it has attracted increasing attention, because it contains an endoperoxide sesquiterpene lactone, artemisinin, which has proved efficacy in killing *Plasmodium falciparum* parasites which cause malaria (Praygod *et al*. 2008). Owing to the increasing resistance to traditional antimalarial drugs like chloroquine and sulphadoxine-pyrimethamine, the demand for artemisinin is increasing enormously (Van Noorden 2010). As artemisinin cannot yet be synthesized chemically in an economically feasible way, at present *A. annua* is the only practical source of this valuable drug. Unfortunately, *A. annua* contains only very small amounts of artemisinin ranging from 0.01 to 0.8% of dry weight (Milhous & Weina 2010), leading to high production costs. Overproduction of key enzymes related

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to biosynthesis of artemisinin, such as HMG-CoA (Aquil *et al*. 2009) or knock down of enzyme activities negatively regulating artemisinin biosynthesis, such as squalene synthase (Zhang *et al.* 2009), all lead to enhanced artemisinin production, which means an important way to reduce the production cost of artemisinin. Because genetic manipulation of *A. annua* provides the most important approach to improve the production of artemisinin, a rapid and reproducible *A. annua* transformation system is of great importance. In the first description of *Agrobacterium tumefaciens* mediated transformation of *A. annua* plants by Vergauwe *et al*. (1996a), different auxin hormone concentrations were tested and vancomycin was used to inhibit *A. tumefaciens* growth, which is rather expensive and which has rather weak activity against *A. tumefaciens* (Vergauwe *et al*. 1998). The penicillin derived antibiotics for decontamination were shown to be more effective, but limited regeneration of shoots from transgenic calli has been observed (Vergauwe *et al.* 1996b). Han *et al.* (2005) used cefotaxime as decontaminating antibiotic, but it reduced callus formation and inhibited shoot induction on leaf explants (Vergauwe *et al*. 1996a).

Up to now, the lack of efficient regeneration of transgenic *A. annua* plants has greatly retarded the progress of molecular research and genetic engineering on *A. annua*. In this work, we described the transformation of *A. annua* stem explants which is completely different from the above reported transformation system using leaves as explants. In addition, we investigated the factors influencing *A. tumefaciens* mediated transformation, for optimization of the *A. annua* transformation and regeneration system.

MATERIAL AND METHODS

Chemical reagents. 6-Benzyladenine (6-BA), α-naphthaleneacetic acid (NAA), 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron, TDZ), kanamycin (Kan), cefotaxime, carbenicillin, rifampicin (Rif) and dimethyl sulphoxide (DMSO) were purchased from Sigma Aldrich Chemical Company (St. Louis, USA). All other chemicals used in this study were of analytical grade obtained from Changsha Chemical Reagent Company (Changsha, China).

***In vitro* grown plant material and culture conditions**. The seeds of *A. annua* were surface sterilized by soaking in 0.5% NaClO for 16 min after immersing in 75% ethanol for 1 min, then they were rinsed three times with sterile distilled water. The sterile seeds were grown on sterile germination medium (MSG, Table 1). Four weeks after germination, the seedlings were transferred to MS medium. Plants were cultivated in a tissue culture room using fluorescent lamps (with a light intensity of 3000 lx) 16 h a day and at a temperature of 25°C with relative humidity of 70%.

Field grown plant material. The seeds of *A. annua* were scattered on wet filter paper, and cultured in a greenhouse. One week after germination, the seedlings were transferred to soil and were cultivated in a greenhouse under the same condition as *in vitro* grown plants.

A. tumefaciens **strain and vectors**. *A. tumefaciens* strain AgL0 (Lazo *et al.* 1991) harbouring the binary vector p35SGUSINT was used in this work. This plasmid contains a β-glucuronidase (GUS) gene with intron which assures that only plant-specific GUS expression is detected in transformed plant tissue. Prior to infection, the bacteria were grown O/N at

Table 1. The composition of culture medium

Medium	Function	Composition
MS	growth medium	MS salts, 2 mg/l glycine, 0.1 mg/l thiamine, 0.5 mg/l pyridoxin, 0.5 mg/l nicotinic acid, 100 mg/l inositol, 3% (w/v) sucrose, 0.8% (w/v) agar, pH 5.8
MSG	germination medium	1/2 MS salts, 2% sucrose, other components are the same to MS, pH 5.8
MSS1	shoot induction medium 1	MS + 0.1 mg/l TDZ, pH 5.8
MSS2	shoot induction medium 2	MS + 0.05 mg/l NAA + 0.5 mg/l 6-BA, pH 5.8
MSE	elongation medium	MS + 0.2 mg/l 6-BA + 30 mg/l Kan + 400 mg/l Cab, pH 5.8
MSR1	rooting medium 1	1/2 MS + *n* mg/l IBA (*n* = 0.05, 0.1, 0.2, 0.5) + 30 mg/l Kan + 200 mg/l Cab, pH 5.8
MSR2	rooting medium 2	1/2 MS + *n* mg/l NAA + (*n* = 0.05, 0.1, 0.2, 0.5) + 30 mg/l Kan + 200 mg/l Cab + 0.1% DMSO, pH 5.8

TDZ – thidiazuron; NAA – α-naphthaleneacetic acid; 6-BA – 6-benzyladenine; Kan – kanamycin; Cab – carbenicillin; DMSO – dimethyl sulphoxide

28°C in liquid LB medium supplemented with selective antibiotics Kan (50 mg/l) and Rif (25 mg/l).

Transformation. Explants were put in 50 ml sterile tubes and 30 ml of a late log growth phase of *A. tumefaciens* culture with 3 µmol of acetosyringone was added. The tubes were gently shaken for 15–20 min, after which the explants were blotted on sterile filter paper and incubated on MS medium supplemented with 100 µmol of acetosyringone per litre medium in dark for 48 h.

Regeneration and selection of transgenic plants. Regeneration of *A. annua* plants was performed according to the procedure described by Lualon *et al.* (2008) with slight modification. Briefly, stem internode segments were cultured on MSS1 medium (Table 1) supplemented with 30 mg/l of Kan and 400 mg/l of carbenicillin for two weeks. When the field grown *A. annua* was used as starting material, the induced shoots were directly transferred to MSE medium. When sterile *in vitro* cultured plant material was used as starting material, the explants were transferred from MSS1 medium to a fresh MSS2 medium supplied with 30 mg/l of Kan and 400 mg/l of carbenicillin. After four weeks the induced shoots were transferred to MSE medium. Once multiple induced shoots appeared on the explants, individual shoots were cut off and transferred to MSR2 medium to induce rooting.

DNA extraction and molecular analysis. DNA was isolated according to the previously reported method (Sangwan *et al.* 1998). The forward primer (5'-GACTGGGCACAACAGACAATCG-3') and the reverse primer (5'-CCAAGCTCTTCAGCAATATCACG-3') were designed based for the *nptII* gene. The 20 µl PCR system included 10 pmol forward primer, 10 pmol reverse primer, 50 ng plant genomic DNA as template, 2 µl 10× Taq DNA polymerase buffer, 0.25 mmol dNTPs, 1 U Taq DNA polymerase (Takara Bio, Inc., Otsu, Japan). Cycling parameters began at 94°C for 5 min, then 30 cycles of denaturation (94°C, 40 s), annealing (60°C, 40 s), and extension (72°C, 30 s), followed by a final extension of 10 min at 72°C in a thermal cycler (Bio-Rad, Hercules, USA). PCR amplification products were analysed by electrophoresis in 1% agarose gel.

GUS staining assay. The leaves of greenhouse-grown plants were analysed for GUS gene expression with X-glucuronide according to Jefferson *et al.* (1987).

RESULTS AND DISCUSSION

Effect of *A. tumefaciens* decontaminators on regeneration

Explants were cultured on MSS1 (internode stems) or MSS2 (leaves and leaf stalks), supplemented either with 500 mg/l carbenicillin or with 500 mg/l cefotaxime. On carbenicillin, 2–3 unhealthy shoots and many friable white calli were induced from 200 leaves or petioles (Figure 1), while no shoots were formed on cefotaxime (data not shown). The results indicated that the *A. annua* leaf was sensitive to both cefotaxime and carbenicillin, which was consistent with previous reports (Vergauwe *et al.* 1996a). However, the cultured stem internodes were resistant to both carbenicillin and cefotaxime; on both media healthy shoots were formed from stem segments (Figure 1). This suggests that stem internodes may be a better source for transformation and regeneration of *A. annua*.

Selection of kanamycin concentration

Explants were cultured on MSS1 medium supplemented with 400 mg/l carbenicillin and different

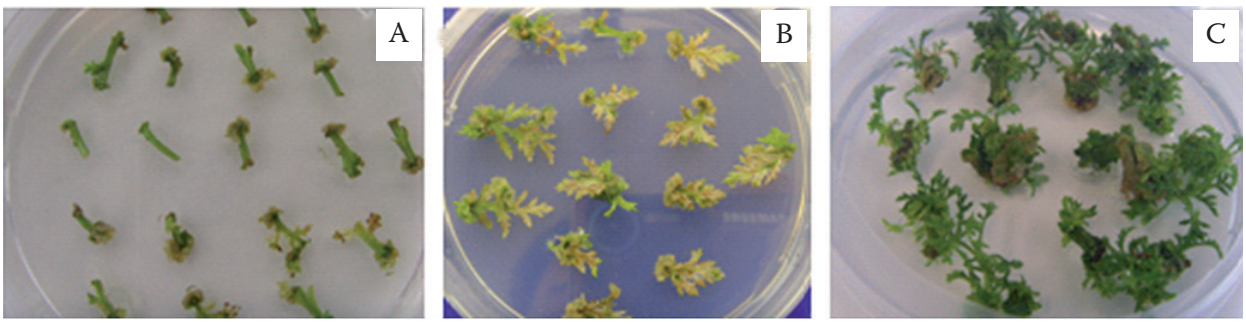

Figure 1. Shoots and calli induced from leaf stalks (A), leaves (B) and stem internodes (C) two weeks after culture on a shoot inducing medium supplied with 500 mg/l carbenicillin

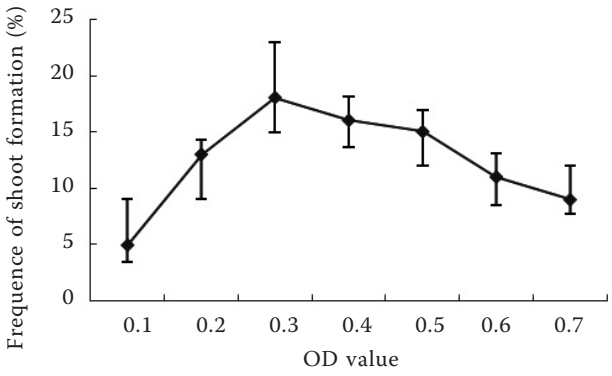

Figure 2. The effect of *Agrobacterium* OD_{600} on transformation efficiency of *Artemisia annua*; transformation efficiency was scored in 45 days post *Agrobacterium* treatment by counting the number of kanamycin-resistant shoots per 100 explants

concentrations of kanamycin ranging from 0 to 100 mg/l. The results indicated that a concentration of 25 mg/l of kanamycin was sufficient to suppress untransformed shoot induction (data not shown). Therefore, in subsequent experiments 30 mg/l of kanamycin was used to select the regeneration of transformed shoots of *A. annua* after *Agrobacterium*-mediated transformation.

Effect of *Agrobacterium* density on transformation

Different *A. tumefaciens* densities were used to infect *A. annua* stem segments, and the number of regenerated Kan resistant shoots per explants was determined after 6 weeks. Figure 2 shows that the transformation frequency initially increases with increasing optical density (OD_{600}) of the bacterial suspension used for transformation, but the transformation frequency declined when OD_{600} was above 0.3. The results therefore suggest that an early exponential growth phase of *A. tumefaciens* is the best stage for infection. A similar correlation between OD_{600} and transformation efficiency has also been observed in other plant transformation systems, such as *Artemisia absinthium* (Mannan *et al.* 2009) and citrus (Dutt & Grosser 2009).

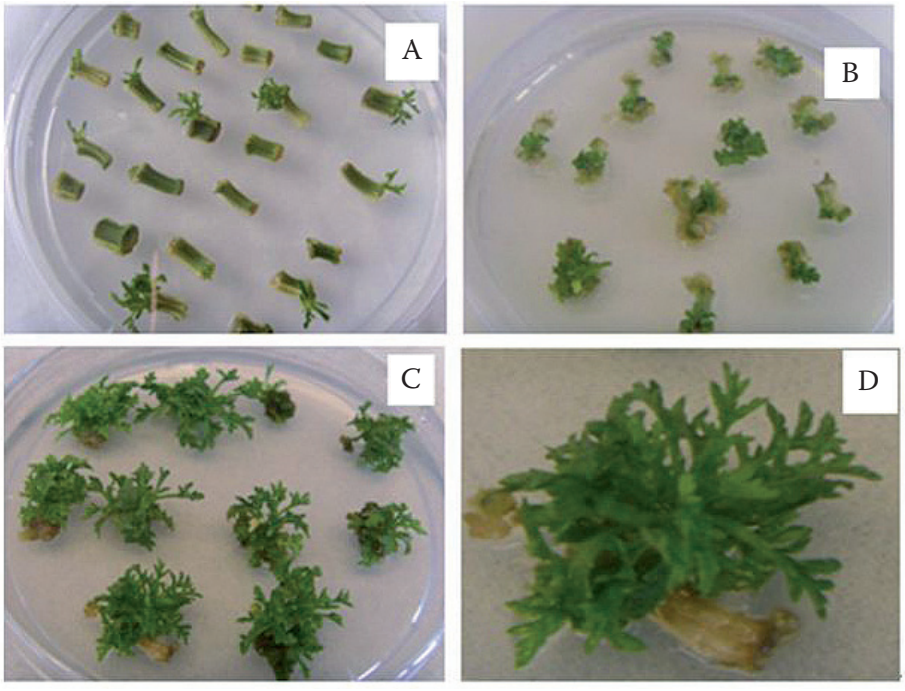

Figure 3. The effect of the physiological state of starting materials on transformation efficiency; A – the stem internodes from field grown *Artemisia annua* formed kanamycin-resistant shoots after two-week culture on regeneration medium containing 25 mg/l Kan and 400 mg/l carbenicillin; B – no kanamycin-resistant shoot was formed in stem internodes from *in vitro* grown *A. annua* after two-week culture on regeneration medium containing 25 mg/l Kan and 400 mg/l carbenicillin; C – kanamycin-resistant shoots induced on stem internodes from *in vitro* grown plant original after 6-week culture on regeneration medium containing 25 mg/l Kan and 400 mg/l carbenicillin; D – five Kan-resistant shoots formed on one explant from C

Table 2. The effect of acetosyringone on transformation efficiency

Acetosyringone concentration (μmol/l)	0	50	100	150
Resistant shoot formation rate (%)	17 ± 3	20 ± 5	45 ± 2	46 ± 3

Effect of acetosyringone concentration on transformation

To test the effect of different concentrations of acetosyringone on the transformation efficiency of *A. annua*, Kan resistant shoots were scored in 6 weeks after transformation and results showed a significant effect of acetosyringone on *A. annua* transformation (Table 2). The optimal concentration was at 100 μmol/l acetosyringone, resulting in 45 kanamycin resistant shoots per 100 explants, compared to 17 transformed shoots per 100 explants when no acetosyringone was used. These results contradict a previous report where acetosyringone did not significantly enhance the transformation efficiency of *A. annua* (Han *et al.* 2005) and it may be due to the fact that different types of explants were used there.

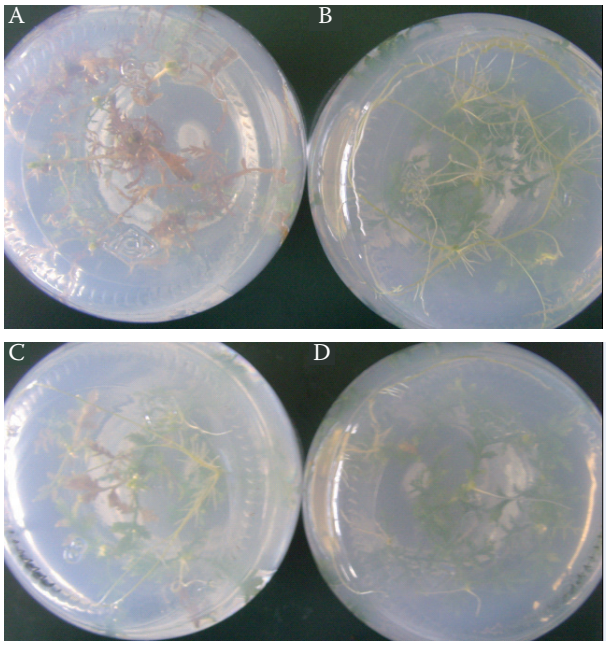

Figure 4. Two-weeks-old wild-type (non-transformed) adventitious shoots induced from stem internodes were excised from explants and placed on a rooting medium supplemented with 25 mg/l of Kan (A) or without Kan (B); two-weeks-old transgenic shoots were cultivated on a selection rooting medium supplemented with (C) or without (D) DMSO

Effects of field and *in vitro* grown plant material on transformation of *A. annua*

All starting materials used in the experiments described above were from *in vitro* grown *A. annua*. In order to analyse the effect of the physiological state of starting materials on transformation efficiency, the stems from *in vitro* grown plants and field grown plants were compared. Results (as shown in Figure 3) indicated that the stem segments from greenhouse grown plant material showed lower regeneration efficiency, with only 20% of explants forming Kan resistant shoots, compared to *in vitro* grown plants with about 45% of explants with regenerated kanamycin-resistant shoots, which may suggest that the required surface sterilization of field grown plant material reduces the adventitious shoot formation. However, kanamycin-resistant shoot regeneration of *in vitro* grown plants took about 6 weeks while regeneration on field grown plants was within two weeks, which implied that the field grown *A. annua* had a stronger rooting ability compared to *in vitro* grown plants (data not shown).

Factors affecting root formation in shoots

After transgenic *A. annua* shoots were obtained, the major obstacle to produce transgenic plants is the

Figure 5. The effect of the physiological age of shoots on rooting; rooting of shoots was scored two weeks after transfer to a rooting medium plus Kan (30 mg/l)

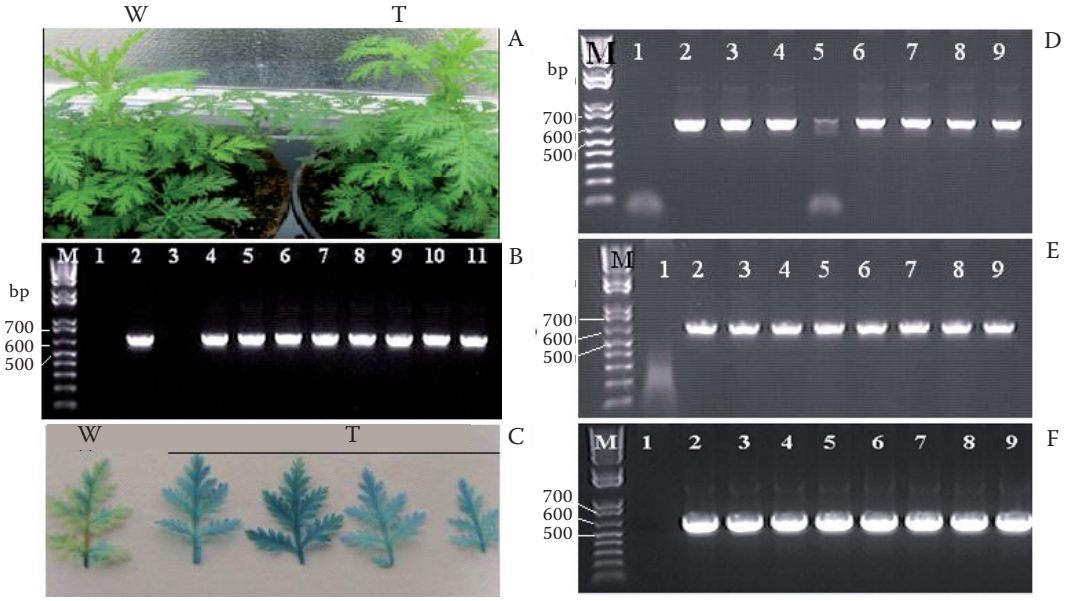

Figure 6. A: phenotype of wild-type (W) and transgenic (T) *Artemisia annua*; B: characterization of putative transgenic seedling T0-1–T0-10 (lane 2–11) and wild-type seedling (lane 1) by PCR amplification of the *nptII* gene; T0-1–T0-10 represented the T0 generation transgenic plant line1–line10, respectively; C: GUS staining of wild-type (W) and transgenic (T) *Artemisia annua* leaves; D: characterization of seedling of the first generation (T1) transgenic *A. annua* (lane 2–9) and wild-type seedling (lane 1) by PCR amplification of the *nptII* gene; T1-1-1–T1-1-4 (lane 2–5) were the progeny of line 1 (T0-1); T1-8-1–T1-8-4 (lane 5–9) were the progeny of line 8 (T0-8); E: characterization of seedling of the second generation (T2) of transgenic *Artemisia annua* (lane 2–9) and wild-type seedling (lane 1) by PCR amplification of the *nptII* gene; T2-1-1-1–T2-1-1-4 (lane 2–5) were the progeny of T1-1-1; T1-8-1-1–T1-8-1-4 (lane 5–9) were the progeny of T1-8-1; F: characterization of putative transgenic *Artemisia annua* seeds from T2 generation plant T2-1-1-1 (lane 2–9) and wild-type seeds (lane 1) by RT-PCR amplification of the *nptII* gene

difficulty in rooting formation under chemical stress produced by Kan and *Agrobacterium* decontaminator. In order to solve the difficulty-to-root problem, different root medium conditions were tested for the efficiency of rooting of transgenic *A. annua* shoots. Results (as indicated in Figure 4A) showed that 99% of wild type shoots formed roots after 3-week cultivation on 1/2 MS medium supplemented with 0.1 mg/l NAA (Table 1), while 25 mg/l of Kan completely inhibited the rooting of non-transgenic shoots. Therefore, 30 mg/l of Kan is used to screen transgenic roots in *A. annua* transformation. The effect of the physiological age of shoots on rooting efficiency was investigated, and results (as shown in Figure 5) showed that the physiological age significantly affected the rooting of kanamycin-resistant shoot in the presence of 30 mg/l of Kan. Two-weeks-old shoots have the highest root inducing capacity, with 25% of the shoots forming roots on 30 mg/l of kanamycin. When the shoots are transferred to a rooting medium after 5 weeks, the root inducing capacity is sharply decreased to 5%.

Interestingly, DMSO significantly enhanced the rooting of transgenic shoots in the presence of Kan (30 mg/l). As shown in Figure 4C and 4D, the highest rooting percentage of 35% was observed on two-weeks-old kanamycin-resistant shoots cultivated on MSR2 medium in combination of 0.1 mg/l NAA with 0.1% (V/V) of DMSO. DMSO enhancement of adventitious root formation was also observed on other species, such as *Senecio greyi* (Hocking *et al.* 1980), *Juniperus communis* (McKinniss 1969), *Hibiscus syriacus* (Whatley *et al.* 1966) and so on. It has been suggested that DMSO acts as a penetrant, increasing NAA uptake into the base of the cutting as well as increasing the movement of endogenous growth regulators and nutrients to the site of root formation (Whatley *et al.* 1966).

Characterization of transgenic *A. annua*

The presence of the transgene in putative transgenic plants was confirmed by PCR amplification of the *nptII* gene on the T-DNA. The expected 632-bp band was detected in 9 out of 10 randomly selected kanamycin resistant plantlets, but it was absent in the negative control (non-transformed) plant (Figure 6B). From the transformed plants (identified by PCR) 100% were found to be positive

in the GUS staining assay (Figure 6C), confirming successful transformation and expression of the 35S-GUS gene into *A. annua*. In order to check if the targeting DNA integrated into the genomic DNA of *A. annua*, the seeds from transgenic *A. annua* germinated on 1/2 MS medium plus 100 mg/l Kan. The kanamycin-resistant seedlings of T1 and T2 generation of transgenic *A. annua* were checked by PCR with *nptII* specific primers, which resulted in the amplification of a 632-bp sequence in the case of the transformants, whereas no amplifications of these sequences could be detected in the wild-type controls (as shown in Figure 6D and E). Results confirmed that the *nptII* gene was integrated into the genomic DNA of *A. annua* and was inherited in progeny. In addition, the RNA from seeds of T2 generation plant was extracted and RT-PCR was applied to characterize the transgenic *A. annua*. And the results (Figure 6F) indicated that the *nptII* gene was expressed in the transgenic seeds, which further implicated that a successful transformation system was established in this work.

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