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

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Abstract


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-pyrimethamidine, 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 (Aquí 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 p3SGUSINT 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

<table>
<thead>
<tr>
<th>Medium</th>
<th>Function</th>
<th>Composition</th>
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<tbody>
<tr>
<td>MS</td>
<td>growth medium</td>
<td>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</td>
</tr>
<tr>
<td>MSG</td>
<td>germination medium</td>
<td>1/2 MS salts, 2% sucrose, other components are the same to MS, pH 5.8</td>
</tr>
<tr>
<td>MSS1</td>
<td>shoot induction medium 1</td>
<td>MS + 0.1 mg/l TDZ, pH 5.8</td>
</tr>
<tr>
<td>MSS2</td>
<td>shoot induction medium 2</td>
<td>MS + 0.05 mg/l NAA + 0.5 mg/l 6-BA, pH 5.8</td>
</tr>
<tr>
<td>MSE</td>
<td>elongation medium</td>
<td>MS + 0.2 mg/l 6-BA + 30 mg/l Kan + 400 mg/l Cab, pH 5.8</td>
</tr>
<tr>
<td>MSR1</td>
<td>rooting medium 1</td>
<td>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</td>
</tr>
<tr>
<td>MSR2</td>
<td>rooting medium 2</td>
<td>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</td>
</tr>
</tbody>
</table>

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′-GACTGGCACAACAGACAATCG-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](image_url). 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.
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 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.

30 mg/l of kanamycin was used to select the regeneration of transformed shoots of A. annua after Agrobacterium-mediated transformation.

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.
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.

<table>
<thead>
<tr>
<th>Acetosyringone concentration (µmol/l)</th>
<th>0</th>
<th>50</th>
<th>100</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistant shoot formation rate (%)</td>
<td>17 ± 3</td>
<td>20 ± 5</td>
<td>45 ± 2</td>
<td>46 ± 3</td>
</tr>
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</table>

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
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 shoots. 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 shoots.

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 (McKiniss 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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