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RESEARCH NOTE

Stable and transient transformation of *Artemisia annua* suspension cells

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Abstract *Artemisia annua* L. is known for its capacity to synthesize artemisinin, a sesquiterpene used as an antimalarial drug. Suspension cell lines have been obtained from this species, but successful genetic transformation of these cell lines has not been reported yet. We established an *A. annua* cell line, AaSA, derived from leaf tissues. Genetic transformation was tested using two different *Agrobacterium tumefaciens* strains bearing a binary plasmid with the neomycin phosphotransferase II as a selectable marker as well as the β-glucoronidase (GUS) reporter gene. Upon selection on kanamycin, stable transgenic calli were obtained and shown to express GUS activity. We also tested whether the AaSA line could be used for transient expression analysis. Both *A. tumefaciens* strains were tested and in both cases, transient expression of GUS was observed after 4–6 days of co-cultivation. This opens the way for metabolic engineering of *A. annua* cells.

Keywords Stable transformation · Transient expression · *Artemisia annua* · Suspension cells

Artemisia annua has been of interest in recent decades because it synthesizes the sesquiterpene lactone artemisinin, a potent antimalarial drug. Artemisinin is produced and stored in the glandular trichomes that cover the aerial parts of the plant (Duke et al. 1994; Olsson et al. 2009). The artemisinin production achieved by the plant is very low. This plant has therefore been largely studied to elucidate the artemisinin biosynthetic pathway and to improve the artemisinin yields.

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Artemisinin production in *A. annua* cells and tissue cultures was investigated by several authors (Paniego and Giulietti 1994; Baldi and Dixit 2008; Caretto et al. 2011; Durante et al. 2011; Fulzele et al. 1991; Heng et al. 2013; Jin and Keng 2013; Wang et al. 2009; Yann et al. 2012). They succeeded in obtaining cell lines which produced small amounts of artemisinin. Despite efforts to improve their artemisinin production, the yields were not relevant for industrial purposes. Nevertheless, those approaches identified chemicals and molecular factors that could have a role in artemisinin biosynthesis. Genetic transformation of *A. annua* suspension cells would be a convenient tool to screen for genes influencing artemisinin biosynthesis and a good alternative to *A. annua* plant transformation which is a long and difficult task (Liu et al. 2010).

To our knowledge, the genetic transformation of *A. annua* suspension cells has not yet been investigated. We therefore examined the possibility of stable and transient genetic transformation of *A. annua* suspension cells using a reporter gene. For this purpose, our first aim consisted of establishing an *A. annua* cell culture. *A. annua* callus induction was performed according to Caretto et al. (2011). Leaf explants from young shoots grown in vitro were placed on solid MSc medium [4.33 g l^{-1} Murashige and Skoog, 3 % (w/v) sucrose, 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.15 mg l^{-1} 6-benzyl amino purine (BAP), and 1 % (w/v) agarose]. After 2 weeks, small calli appeared. Calli were subcultured on fresh medium after 6 weeks. After duplication, they were cultured either in light [16 h light (50 µmol m^{-2} s^{-1}), 24 °C] or in dark conditions. No callus was able to grow under dark conditions. Friable calli growing in the light were transferred to liquid medium. For calli that were able to grow in liquid medium, we tested four different media: MS ½, MS, MSa, and B5 (Table 1). The best growth rate was

Table 1 Media tested for the *A. annua* cell cultures

Medium	Composition
B5	3.16 g l^{-1} Gamborg Medium (B5), 20 g l^{-1} sucrose, 75 µg l^{-1} BAP and 1 mg l^{-1} 2,4 D, pH 5.8 (KOH)
MS ½	2.2 g l^{-1} Murashige and Skoog, 3 % (w/v) sucrose, NAA 250 µg l^{-1} 2,4-D and 250 µg l^{-1} BAP, pH 5.8 (KOH)
MS	4.33 g l^{-1} Murashige and Skoog, 3 % (w/v) sucrose, 1 mg l^{-1} 2,4-D and 100 µg l^{-1}BAP, pH 5.8 (KOH)
MSa	4.33 g l^{-1} Murashige and Skoog, 3 % (w/v) sucrose, 50 mg l^{-1} myo-inositol, 2,5 mg l^{-1} thiamine, 0.2 g l^{-1} KH_2PO_4, 2 mg l^{-1} 2,4-D, 165 µg l^{-1} NAA and 171 µg l^{-1} BAP, pH 5.8 (KOH)
MSa#	4.33 g l^{-1} Murashige and Skoog, 3 % (w/v) sucrose, 10 mM glucose, 50 mg l^{-1} myo-inositol, 2,5 mg l^{-1} thiamine, 0.2 g l^{-1} KH_2PO_4, 2 mg l^{-1} 2,4-D, 165 µg l^{-1} NAA and 171 µg l^{-1} BAP, pH 5.3 (KOH)

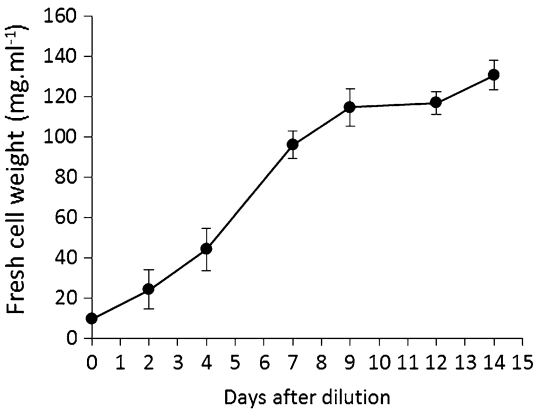

Fig. 1 Growth curve of the *A. annua* suspension cell line AaSA. For each point, the fresh cell weight was measured after filtration of 2 ml of culture. This experiment was carried out in triplicates. Means and confidence intervals are shown

obtained for the MSa medium, which allowed for the subculture of calli every 2 weeks compared to every 4 weeks for the other media tested. During successive subculturing, some of the cell lines were gradually lost due to too slow growth. Cell cultures were diluted every 2 weeks by transferring 10 ml from the old culture into 40 ml of MSa medium. One cell line, AaSA, which formed small cell aggregates, was selected for further study. Figure 1 shows a growth curve of this line.

We investigated the capacity of the AaSA cells to be genetically transformed using two *A. tumefaciens* strains: LBA4404virG (van der Fits et al. 2000) and AGL1 (Lazo et al. 1991). Both were transformed with the binary vector pPZP-RCS2-En2pPMA4-GusVenus-tNOS-nptII which contains, between the T-DNA right and left borders, the expression cassette of the *GUS-VENUS* hybrid reporter and the marker gene *nptII*, conferring resistance to kanamycin (Navarre et al. 2010).

Cells of zero- (just after subculturing), 4-, 6- or 8-day-old cultures of *A. annua* AaSA were washed in MSa# medium (MSa medium supplemented with 10 mM glucose, pH 5.3) and resuspended in MSa# medium supplemented with 50 µM acetosyringone. In the meantime, *A. tumefaciens* strains were grown for 16 h at 28 °C in 10 ml 2YT (1.6 % (w/v) tryptone, 1 % (w/v) yeast extract, 0.5 % (w/v) NaCl). For the LBA4404 strain, 40 mg l^{-1} gentamycin, 20 mg l^{-1} rifampicine and, for plasmid selection, 100 mg l^{-1} spectinomycin were added. For the AGL1 strain, 40 mg l^{-1} carbenicillin and, for plasmid selection, 100 mg l^{-1} spectinomycin were added. A volume of the culture corresponding to an OD_{600} of 1 in 1 ml was centrifuged, and the cells were resuspended in 1 ml of MSa#. The duration of the co-cultivation of *A. annua* and *A. tumefaciens* cells was fixed to 48 h under light and agitation conditions. Both the LBA4404virG and AGL1 *A. tumefaciens* strains were tested. To assess the optimal cell culture age, we made the hypothesis that the appropriate conditions for transient expression would be convenient for stable transformation. We therefore performed a GUS assay after 48 h of co-cultivation. Cultures at days 6 and 8 after subculturing showed the highest GUS-staining for both *A. tumefaciens* strains (Fig. 2).

For stable transformation, 4 ml of 8-days-old *A. annua* AaSA suspension were mixed with 0, 50, 100, 200, or 300 µl of the *A. tumefaciens* LBA4404virG suspension in 6-well plates (Greiner) and co-cultured for 2 h at 25 °C in light conditions without agitation followed by 48 h with agitation. The cells were then centrifuged and washed twice with MSa, then washed with MSa CCK20 (MSa medium containing 20 µg ml^{-1} kanamycin, 500 µg ml^{-1} cefotaxin, and 400 µg ml^{-1} carbenicillin). The cells were allowed to sediment and were resuspended in 2 ml of MSa CCK20 and spread on solid MSa CCK20 (0.8 % (w/v) agar). The plates were incubated at 24 °C in light conditions. After 25 days, growing calli were transferred twice, at 3-week intervals, on MSa CCK20 and then on MSa K20 (MSa containing 20 µg ml^{-1} kanamycin). An example of growing calli is shown in Fig. 3a. Finally, calli were transferred to liquid MSa K20 for expression screening. All transformed calli tested showed GUS activity (Fig. 3b). GUS expression was unchanged after 16 transfers (8 months) to a fresh medium, indicating that the transformants were stable.

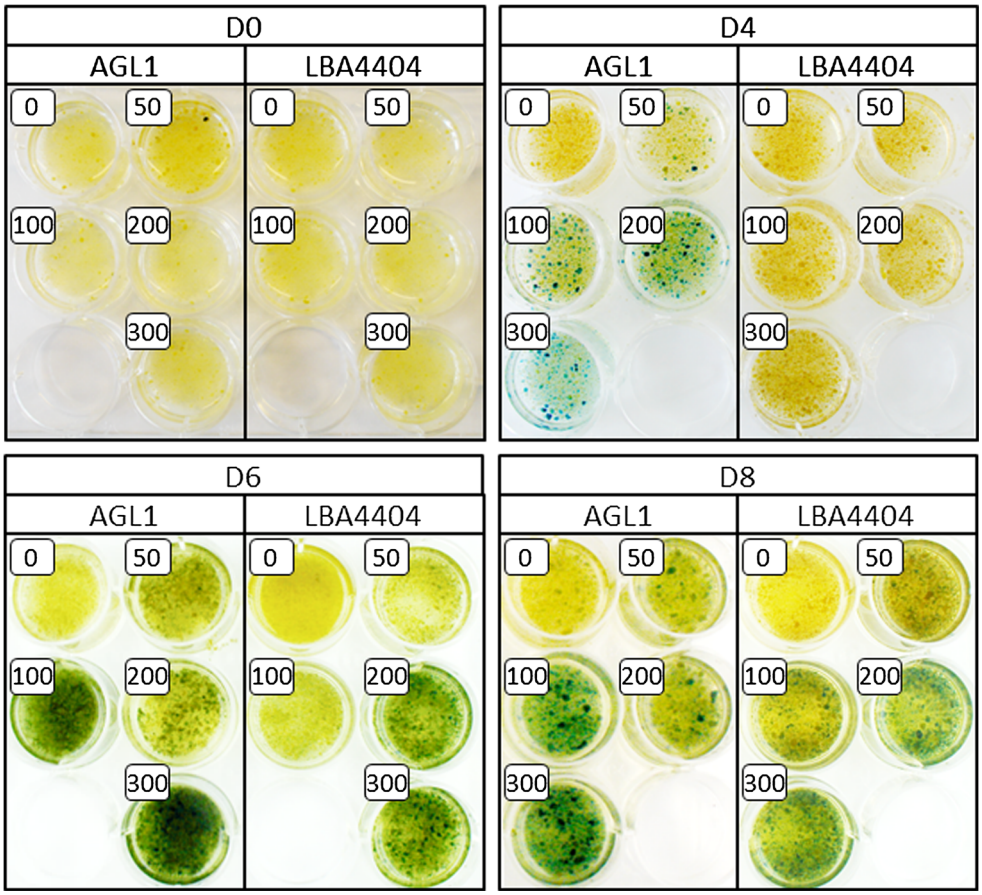

Fig. 2 Transient expression in *A. annua* suspension cells. *A. annua* cells were infected with *A. tumefaciens* lines AGL1 or LBA4404 containing the *GUSVenus* expression cassette. 4 ml of *A. annua* AaSA culture at 0, 4, 6, and 8 days after sub-culturing were mixed with 0, 50, 100, 200, or 300 µl of *A. tumefaciens* culture at an OD_{600} of 1. GUS staining was performed 48 h later

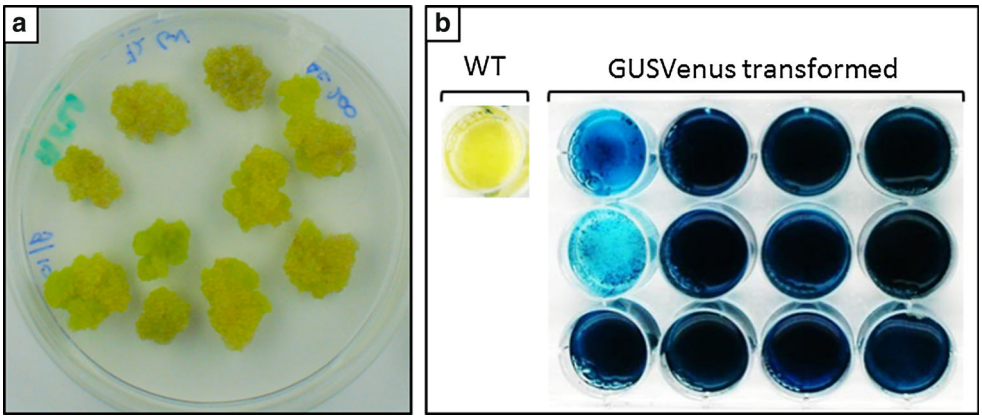

Fig. 3 GUS activity of *GusVenus* transformed *A. annua* calli. **a** Calli obtained from the AaSA line after transformation with the *GUSVenus* construct and selection on kanamycin medium. **b** Twelve calli were tested for GUS activity. *A. annua* suspension cells were transferred to a reaction medium containing 34.2 mM Na_2HPO_4, 15.8 mM NaH_2PO_4 (pH 7.0), 0.1 % Triton X100 (v/v), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. The cells were incubated at 37 °C in dark conditions until the appearance of blue staining. *WT* wild-type

Transient expression upon transformation by *A. tumefaciens* depends on the T-DNA copies transferred to the plant cell nucleus but not yet integrated into the genome. Transient expression has the major advantage of being fast since no selection of transformed calli is necessary. In addition, transient expression might give higher expression than that seen during stable transformation, especially if the expressed gene affects cell growth and survival. Since GUS activity was detected prior to growing cells on selective medium (Fig. 2), we decided to optimize the protocol for transient gene expression. 4 ml of *A. annua* 8-days-old AaSA culture were co-cultured with 50, 100, or

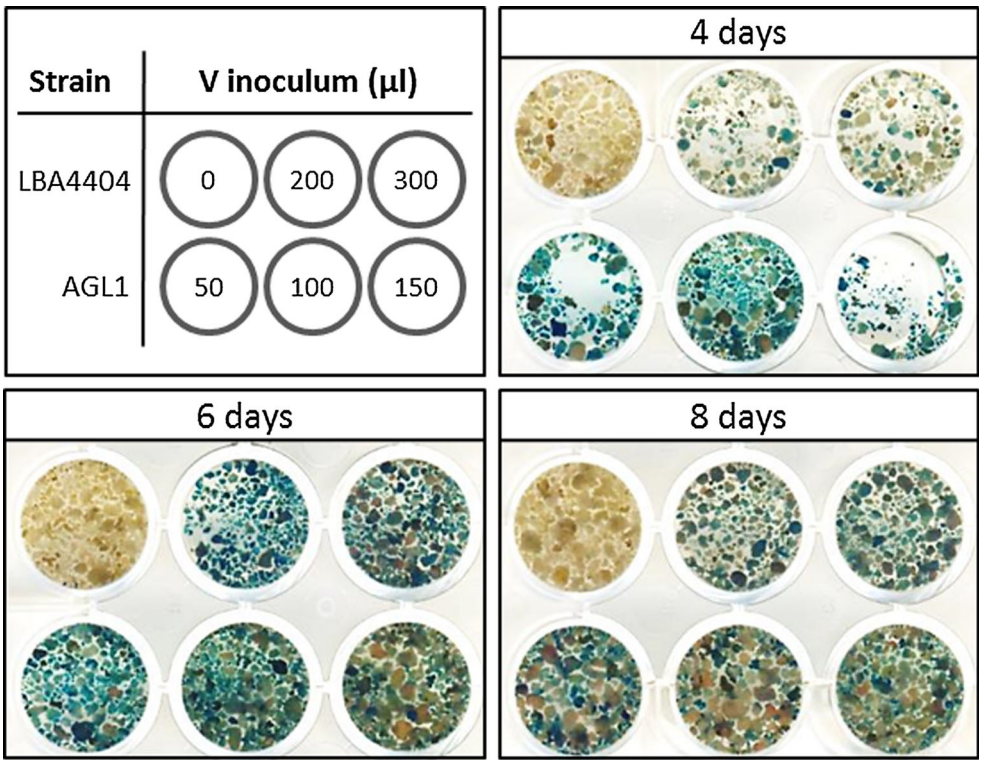

Fig. 4 GUS staining of *A. annua* suspension cells transiently expressing the *GUSVenus* reporter gene. *A. annua* cells were mixed with an LBA4404 or AGL1 *A. tumefaciens* line containing the *GUSVenus* expression cassette. 4 ml of *A. annua* culture (day 7) were mixed with 200 or 300 µl of the LBA4404 line or with 50, 100, or 150 µl of the AGL1 line. Bacterial cultures had an OD_{600} of 1. GUS staining was assayed after co-cultivation for 4, 6, and 8 days

150 µl of the *A. tumefaciens* AGL1 suspension, or, 200 or 300 µl of the *A. tumefaciens* LBA4404virG suspension in 6-well plates (Greiner). Co-cultures were incubated in light conditions at 25 °C for 2 h without agitation followed by 4–8 days of incubation with agitation. A GUS staining assay was performed on each co-culture (Fig. 4). The AGLI line showed the highest staining after 4 days of co-cultivation while this stage was delayed to the sixth day for LBA4404virG.

In this report, we demonstrated the possibility of stable and transient genetic transformation of *A. annua* suspension cells mediated by *A. tumefaciens* infection. The AGL1 and LBA4404virG strains were both able to transfer their T-DNA to *A. annua* cells. Since our purpose was to demonstrate that genetic transformation was possible, we did not perform metabolic analyses to determine whether AaSA cells synthetize artemisinin precursors. If this was the case, molecular biology tools could be used to obtain a better understanding of the metabolic pathway leading to the synthesis and transport of artemisinin and eventually for metabolic engineering. If it turns out that the AaSA line is not appropriate for this purpose, one could shift to other lines, which have already been shown to be active in the artemisinin pathway, either constitutively or after induction. Indeed, since we did not encounter any major problems with the line tested, it can be expected that other *A. annua* lines would be prone to be transformed as well.

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