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# *IN VITRO* AND *IN PLANTA AGROBACTERIUM TUMEFACIENS* - MEDIATED TRANSFORMATION OF *ARABIDOPSIS THALIANA*

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Cell transformation of *Arabidopsis thaliana* (L.) Heynh. with *Agrobacterium tumefaciens* (LBA4404 strain) was performed in two ways: *in vitro* - by the transformation of root and stem explants - and *in planta* - by the transformation of whole plants in the greenhouse. *Agrobacterium* contained the binary plasmid pBI121 or its constructs with the fragments of carrot DC-8 promoter. The results show that transformation efficiency *in vitro* is influenced by the age and type of explant. It was not possible to induce transgenic calli on explants of 5 week old *Arabidopsis* roots, although it was possible on explants of 3 week old roots. Although green calli grew on *Arabidopsis* stem explants, transgenic shoots did not develop. When using transformation *in vitro*, from seed to mature primary transformant it took 15 to 16 weeks and for *in planta* transformation it took 19 to 20 weeks.

## Introduction

Gene transfer to plants has developed into an important tool for studies in basic plant biology, allowing the analysis of gene structure and regulation (P o t r y k u s 1990). Although it has certain limitations, the introduction

of foreign DNA into plant cells by *Agrobacterium tumefaciens*-mediated transformation is still the most frequent way of obtaining transgenic plants (B i n n s 1990).

*Arabidopsis thaliana* (L.) Heynh. due to its small genome, short generation time and easy maintenance in the laboratory and in the greenhouse is a very suitable experimental model for research into the molecular and genetic level of plant physiology, biochemistry and development (M e y e r o w i t z 1989). Therefore a considerable amount of knowledge about the genetics and biology of this plant species has been gained, which has resulted in enhanced research interest in *Arabidopsis*. As this plant is also suitable for *in vitro* manipulation, a number of protocols for different tissue culture methods have been developed (K a r e s c h et al. 1991; W u et al. 1992). It is possible successfully to introduce foreign genes into *Arabidopsis* cells by *Agrobacterium tumefaciens*-mediated transformation (S c h m i d t and W i l l m i t z e r 1988; V a l v e k e n s et al. 1988). Recently, a stable genetic transformation of *Arabidopsis* by *in planta* inoculation of *Agrobacterium* was also achieved (C h a n g et al. 1990; C h a n g et al. 1994; K a t a v i c et al. 1994).

In our experiments we performed both *in vitro* and *in planta Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. This research was a part of a bigger project in which we analysed the activity of a DC-8 promoter (from a carrot embryogenic gene DC-8; F r a n z et al. 1989) in transgenic *Arabidopsis* plants. Here we present the results of both methods of transformation and discuss their advantages and disadvantages.

## M a t e r i a l s a n d M e t h o d s

*Plant material.* In our experiments we used *Arabidopsis thaliana* (L.) Heynh. seeds, ecotype Columbia (R e d e i 1970). For transformation *in planta* besides ecotype Columbia we used also ecotype Wassilewskija.

*Bacterial material.* For transformation we used *Agrobacterium tumefaciens*, LBA4404 strain (H o e k e m a et al. 1985). The vector was a binary plasmid pBI121 (Clontech Laboratories, Inc.) with the 35S promoter (800 bp of 35S promoter from cauliflower mosaic virus, CaMV) and the coding sequence of a bacterial gene for beta-glucuronidase (GUS, 1.9 kbp) (Fig. 1). We also used bacteria with pBI121 constructs (provided by R. S u n g, University of California, Berkeley) in which the 35S promoter was replaced with the carrot DC-8 promoter (i.e. its 2600, 505 and 305 bp fragments; G o u p i l et al. 1992; Fig. 1).

*Tissue culture conditions and media.* In general we used the methods described by S t r e e t (1977). We autoclaved media for 20 min at 120°C and 103,4 MPa. Hormones and antibiotics were filter-sterilised and added to the medium after the autoclaving and cooling. The used media content is shown in Table 1.

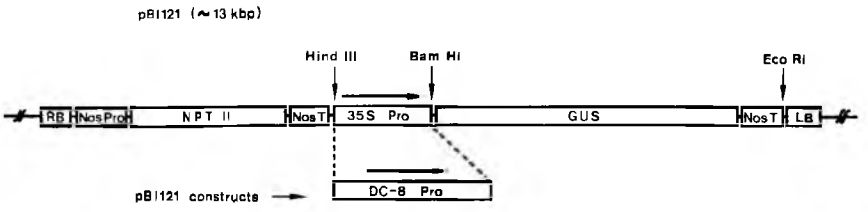


Fig. 1. Structure of a binary plasmid pBI121 and its constructs in which the 35S promoter was replaced with the carrot DC-8 promoter (or fragments of it). Adapted from J e f f e r s o n et al. (1987) and G o u p i l et al. (1992). Not drawn to scale.
RB,LB - right and left T-DNA border sequence; NPT II - gene for neomycin phosphotransferase (responsible for kanamycin resistance); NOS Pro, NOS T - promoter and terminator from gene for nopaline synthase; GUS - bacterial gene for beta-glucuronidase; 35S pro - 35S promoter from cauliflower mosaic virus; DC-8 pro - promoter from carrot DC-8 gene.

*Sterilisation and germination of Arabidopsis seeds.* We surface-sterilised *Arabidopsis* seeds for 2 min in 70 % ethanol and then 40 min in 0.5 % NaOCl. Afterwards we rinsed the seeds 5 times with sterile distilled water and placed them on Petri dishes with a germination medium (GM, Table 1), 50 to 100 seeds per dish. We vernalized the seeds for 4 days at 4°C. The seeds germinated at 20°C in a 9 hour light (70 to 100 $\mu Es^{-1} m^{-2}$)/15 hour dark cycle. In experiments where we tested the seeds for kanamycin resistance (after *in planta* transformation) we mixed seeds - after sterilisation and rinsing - with warm 0.5 % agarose and poured them on the solidified GM with the addition of 50 $mgl^{-1}$ kanamycin.

*Root and stem explant transformation.* We performed *Arabidopsis* root explant transformation according to the protocol suggested by V a l v e k e n s et al. (1988). Roots of sterile germinated plants were first incubated on a callus inducing medium (CIM, Table 1) for 3 days at 20°C in a short-day light cycle. Then we mixed root explants with a saturated bacterial suspension and cut them into pieces (0.5 cm long) and grouped them on the CIM. After 48 hours of cocultivation with agrobacteria we washed the explants with a liquid B5 medium supplemented with 500 $mgl^{-1}$ carbenicillin for 30 min on a shaker (80 rpm). Then we placed the root explants on a shoot inducing medium (SIM, Table 1) supplemented with 500 $mgl^{-1}$ carbenicillin and 50 $mgl^{-1}$ kanamycin. The root explants were then incubated in growth chambers at 25°C. We transferred the explants to fresh medium (SIM) every 7 days. We isolated a green callus tissue (which had formed on the root explants) from the root tissue within 3 weeks of their appearance and subcultivated them on fresh SIM with antibiotics. We transferred green shoots to an elongation medium (EM, Table 1) supplemented with 50 $mgl^{-1}$ kanamycin in Magenta boxes (4 shoots per box). When the shoots were strong enough they were subcultivated on a root inducing medium (RIM, Table 1). After the roots developed, we gradually adapted transgenic plants to greenhouse conditions. We transfor-

med *Arabidopsis* stem explants in the same way as the root explants. We used stems of sterile germinated plants cut in 0.5 to 1 cm long segments, which were put on the medium individually, not in clusters like the root explants.

Table 1. Composition of the tissue culture media used during the transformation of *Arabidopsis* explants and selection and regeneration of transgenic plants. All media contained 1 % sucrose and were solidified with 0.8 % agar when needed.

| MEDIUM | BASIC MEDIUM | PLANT GROWTH REGULATORS |
|---|---|---|
| GM (germination medium) | MS | without growth regulators |
| CIM (callus inducing medium) | B5 | 0.5 $mgl^{-1}$ 2,4 D<br>0.05 $mgl^{-1}$ kinetin |
| SIM (shoot inducing medium) | B5 | 5 $mgl^{-1}$ 2iP<br>0.15 $mgl^{-1}$ IAA |
| EM (shoot elongating medium) | MS | 1 $mgl^{-1}$ IBA |
| RIM (root inducing medium) | B5 | 0.7 $mgl^{-1}$ kinetin<br>0.7 $mgl^{-1}$ NAA |

(B5 - Gamborg et al. 1968; MS - Murashige and Skoog 1962)
2,4 D - 2,4-Dichloro-phenoxyacetic acid
kinetin - 6-Furfurylaminopurine
2iP - 6-(gama,gama-dimethylallylamino)purine
IAA - Indole-3-acetic acid
IBA - Indole-3-butyric acid
NAA - 1-Naphtaleneacetic acid

*Transformation in planta.* For *in planta* transformation we used young and healthy *Arabidopsis* plants (ecotypes Columbia and Wassilewskija) which were grown in the greenhouse at 25°C in a long-day light cycle. After the plants bolted up to 2 to 3 cm we carefully removed all the bolts with a scalpel and inoculated the wound with 20 µl of saturated bacterial suspension. Control plants were inoculated with 20 µl of sterile YEB medium (0.1 % yeast extract, 0.5 % beef extract, 0.5 % Bacto-peptone, 0.5 % sucrose and 0.05 % Mg $SO_4$ x $H_2O$). We inoculated 50 plants of each ecotype, while control groups consisted of 20 plants. After 10 days, when the plants bolted again, we repeated the procedure with a fresh bacterial suspension. After the plants bolted again we let them mature and then collected the seeds from each plant separately.

*Cultivation of bacteria.* We kept the agrobacteria in 30 % glycerol at -70°C. Prior to transformation we inoculated bacteria on solidified YEB medium (with the addition of 1.5 % agar) with 50 $mgl^{-1}$ kanamycin and incubated them at 28°C for 24 hours. We inoculated individual colonies separately in liquid YEB medium with kanamycin 48 hours before the transformation. Just

before the infection, we pooled suspensions originating from individual bacterial colonies (with the same plasmid construct) and this integrated suspension was used for the transformation.

## Results

We transformed root and stem explants of *Arabidopsis thaliana* with *Agrobacterium tumefaciens*, LBA4404 strain with binary plasmid pBI121 (containing the bacterial gene for beta-glucuronidase under the control of the 35S promoter) or its constructs in which the 35S promoter was replaced with the carrot DC-8 promoter, i.e. its 2600, 505 and 305 bp fragments (Fig. 1).

Prior to cocultivation with agrobacteria, we incubated root and stem explants for 3 days on callus inducing medium (CIM, Table 1). This treatment resulted in an intense development of root hairs on the root explants. Ten days after the infection green swellings of transgenic callus tissue were visible on the explants. At the same time, untransformed parts of the tissue became yellow because of the antibiotics. After 3 weeks we separated green calli from the explant tissue and subcultivated them on fresh SIM with antibiotics. We transferred green shoots, which regenerated from callus tissue after 10 to 14 days, to Magenta boxes; first on elongating medium (EM, Table 1) with the addition of kanamycin (50 $mgl^{-1}$) and then to root inducing medium (RIM, Table 1), also with antibiotic. It took 3 to 5 weeks for roots to develop.

Table 2. Efficiency of *Arabidopsis* root explant transformation with *Agrobacterium tumefaciens*, LBA4404 strain, with pBI121 plasmid and its constructs which had the carrot DC-8 gene promoter or different fragments of it.

| Plasmid construct | Age of roots at moment of inoculation | Number of inoculated root explants | Root explants which developed green callus tissue | | Green calli which regenerated shoots | |
|---|---|---|---|---|---|---|
| | | | number | % | number | % |
| pBI121(Δ35S) DC-8/2600 | 3 weeks | 411 | 6 | 1.5 | 2 | 33.3 |
| pBI121(Δ35S) DC-8/2600 | 5 weeks | 725 | 0 | 0.0 | 0 | 0.0 |
| pBI121(Δ35S) DC-8/505 | 3 weeks | 482 | 22 | 4.6 | 7 | 31.8 |
| pBI121(Δ35S) DC-8/305 | 3 weeks | 425 | 24 | 5.6 | 2 | 8.3 |
| pBI121 | 3 weeks | 463 | 32 | 6.9 | 5 | 15.6 |
| pBI121 | 5 weeks | 400 | 0 | 0.0 | 0 | 0.0 |
| control (Columbia wild type) | 3 weeks | 390 | 0 | 0.0 | 0 | 0.0 |

The transformation efficiency for the root explants is shown on Table 2. We did not notice transgenic callus tissue on the inoculated explants of 5 week old roots even after 4 months of regular weekly transfer to fresh SIM.

Results of stem explant transformation are shown in Table 3. Green calli that developed on the explants 10 days after inoculation (and which we separated out after 3 weeks and subcultivated on SIM with antibiotics) failed to regenerate shoots even after 3 months of regular weekly subcultivation on fresh medium.

Table 3. Results of *Arabidopsis* stem explant transformation with *Agrobacterium tumefaciens*, LBA4404 strain, with pBI121 plasmid and its constructs which had the carrot DC-8 gene promoter or different fragments of it.

| Plasmid construct | Age of stems at moment of inoculation | Stem of inoculated stem explants | Stem explants which developed green callus tissue | | Green calli which regenerated shoots | |
|---|---|---|---|---|---|---|
| | | | number | % | number | % |
| pBI121(Δ35S) DC-8/2600 | 3 weeks | 213 | 7 | 3.3 | 0 | 0.0 |
| pBI121(Δ35S) DC-8/505 | 3 weeks | 196 | 10 | 5.1 | 0 | 0.0 |
| pBI121 | 3 weeks | 202 | 10 | 4.9 | 0 | 0.0 |
| control (Columbia wild type) | 3 weeks | 180 | 0 | 0.0 | 0 | 0.0 |

For *in planta* transformation we used *Agrobacterium tumefaciens*, LBA4404 strain with a construct of pBI121 plasmid which contained the DC-8 promoter (Fig. 1). Because the incorporation of the chimeric gene into the plant cells results in kanamycin resistance, we tested seeds of the individual plants by inoculating them on solidified MS medium with the addition of 50 $mgl^{1}$ kanamycin. We tested 1500 to 2500 seeds per plant. We transferred potential transformants - seedlings resistant to kanamycin - to a fresh medium with antibiotic every 2 weeks because some of them expressed their sensitivity to kanamycin only after a few weeks. Most untransformed seedlings died during the first 5 weeks on the medium with the antibiotic. All seeds tested from control plants were kanamycin sensitive. The transformation efficiency was 6 % for ecotype Columbia (3 independent transformants on 50 inoculated plants) and 4 % for ecotype Wassilewskija (2 independent transformants on 50 inoculated plants).

## Discussion

In our experiments, transgenic calli did not develop on the root explants of 5 week old roots (Table 2), which shows that the age of the explant plays an important role in successful transformation. Schmidt and Willmitzer (1988) noticed in their experiments that different ages of *Arabidopsis* leaf explants did not have an influence on the transformation efficiency of ecotype C24 but were important for ecotype Wassilewskija (in their experiments the highest transformation efficiency was obtained on explants of 2 week old leaves). The type of organ used for transformation in our experiments also had an influence on transformation efficiency. So, although stem explants (transformed with any of the three plasmids) developed green callus tissue on the medium with kanamycin they failed to produce shoots (Table 3), even after 3 months in culture. Sangwan et al. (1992) stated that plant cell competence is important for successful transformation with agrobacteria. This competence can be achieved with a suitable pre-treatment with plant growth regulators. In order to achieve competence we incubated *Arabidopsis* explants for 3 days on a medium supplemented with 0.5 $mgl^{-1}$ 2,4 D and 0.05 $mgl^{-1}$ kinetin (CIM, Table 1). Valvekens et al. (1988) showed that such pre-treatment was useful for more intense shoot regeneration.

The efficiency of *in planta* transformation of *Arabidopsis* (screened as a resistance to kanamycin in seeds of primary transformants) was 4 % for Wassilewskija ecotype and 6 % for Columbia ecotype. It has been shown that transformation frequencies for *in planta* transformation varied considerably even between separate experiments within one laboratory (Katavic et al. 1994). Same authors obtained 9.6 % primary transformants in one laboratory and 3.6 % in another. Chang et al. (1994) in their experiments with *in planta* transformation achieved a transformation frequency of 5.5 %. In their previous experiments (Chang et al. 1990), in which they tested beta-glucuronidase activity in *Arabidopsis* flowers after *in planta* transformation with pBI121 plasmid, more than 30 % of tested inflorescences showed enzyme activity. Such a high percentage might be due not only to the activity of the gene integrated into the plant cell genome but to the bacteria still present in intercellular spaces.

When transformation *in planta* is concerned we needed 19 to 20 weeks from germination of seed (in order to grow the plant on which we would perform the transformation) to adult primary transformant. Such a long time was needed because it took almost 5 weeks for some of germinating seeds (progeny of inoculated plants, potential primary transformants) to express their sensitivity to kanamycin. On the other hand, in root explant transformation we obtained primary transformants in 15 to 16 weeks.

The advantages of *in planta* transformation in comparison with the root explant transformation are simplicity of method and the possibility of

transformation of a high number of plants. But after they mature a huge number of seeds have to be screened for kanamycin resistance, which is rather expensive and time-consuming. By *in vitro* transformation it is possible to obtain primary transformants faster but the critical moments are the rooting of transgenic shoots (which can take up to 5 weeks) and transfer to the greenhouse, during which many transgenic plants can be lost.

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## R e f e r e n c e s

*Binns, A.N.*, 1990: *Agrobacterium* - mediated gene delivery and the biology of host range limitations. Physiol. Plant. *79*, 135-139.

*Chang, S.-S., S.-K. Park, H.-G. Nam*, 1990: Transformation of *Arabidopsis by Agrobacterium* inoculation on wounds. In: Abstracts of the Fourth International Conference of Arabidopsis Research, Vienna, pp. 28.

*Chang, S. S., S. K. Park, B.C. Kim, B.J. Kang, D.U. Kim, H.G. Nam*, 1994: Stable genetic transformation of *Arabidopsis thaliana* by *Agrobacterium* inoculation ***in planta***. Plant J. *5(4)*, 551-558.

*Franz, G., P. Hatzopoulos, T.J. Jones, M. Krauss, Z.R. Sung*, 1989: Molecular and genetic analysis of an embryonic gene, DC-8, from *Daucus carota* L. Mol. Gen. Genet. *218*, 143-151.

*Gamborg, O.L., R.A. Miller, K. Ojima*, 1968: Plant cell cultures : I. Nutritional require ments of suspension cultures of soybean root cells. Exp. Cell Res. *50*, 151-158.

*Goupil, P., P. Hatzopoulos, G. Franz. F.D. Hempel, R.You, Z.R. Sung*, 1992: Transcriptional regulation of a seed-specific carrot gene, DC8. Plant Mol. Biol. *18*, 1049-1063.

*Hoekema, A., M. Van Haren, A. Fellinger, P. Hooykaas, R. Schilperoort*, 1985: Non-oncogenic plant vectors for use in the agrobacterium binary systems. Plant Mol. Biol. *5*, 85-89.

*Jefferson, R.A., T.A. Kavanagh, M.W. Bevan*, 1987: GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. *6(13)*, 3901-3907.

*Karesch, H., R. Bilang, I. Potrykus*, 1991: *Arabidopsis thaliana*: protocol for plant regeneration from protoplasts. Plant Cell Rep. *9*, 575-578.

*Katavic, V., G.W. Haughn, D. Reed, M. Martin, Lj. Kunst*, 1994: In planta transformation of *Arabidopsis thaliana*. Mol. Gen. Genet. *245*, 363-370.

*Meyerowitz, E.M.*, 1989: Arabidopsis, a useful weed. Cell *56*, 263-269.

*Murashige, T., F. Skoog* 1962: A revised medium for the rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. *15*, 473-497.

*Potrykus, I.*, 1990: Gene transfer to plants: assessment and perspectives. Physiol. Plant. *79*, 125-134.

*Redei, G.P.*, 1970: *Arabidopsis thaliana* (L.) Heynh. A review of the genetics and biology. Bibliogr. Genetica *20*, 1-151.

*Sangwan, R.S., Y. Bourgeois, S. Brown, G. Vasseur, B. Sangwan-Norreel,* 1992: Characterization of competent cells and early events of *Agrobacterium* -mediated genetic transformation in Arabidopsis thaliana. Planta *188*, 439-456.

*Schmidt, R., L. Willmitzer,* 1988: High efficiency *Agrobacterium tumefaciens* - mediated transformation of *Arabidopsis thaliana* leaf and cotyledon explants. Plant Cell Rep. *7*, 583-586.

*Street, H.E.,* 1977: Plant Tissue and Cell Culture (Second ed.), Blackwell Scientific Publications. Oxford, London, Edinburgh, Melbourne

*Valvekens, D., M. Van Montagu, M. Van Lijsebettens,* 1988: *Agrobacterium tumefaciens* - mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc. Natl. Acad. Sci. USA *85*, 5536-5540.

*Wu, Y., G. Haberland, C. Zhou, H.-U. Koop,* 1992: Somatic embryogenesis, formation of morphogenetic callus and normal development in zygotic embryos of *Arabidopsis thaliana in vitro.* Protoplasma *169*, 89-96.

## SAŽETAK

### *IN VITRO* I *IN PLANTA* TRANSFORMACIJA BILJAKA VRSTE *ARABIDOPSIS THALIANA* (L.) HEYN., POMOĆU BAKTERIJE *AGROBACTERIUM TUMEFACIENS*

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U radu je izvršena transformacija eksplantata korjenčića i stabljike (*in vitro* transformacija), te kompletnih biljaka (*in planta* transformacija) uročnjaka *Arabidopsis thaliana* (L.) Heynh. bakterijom *Agrobacterium tumefaciens*, soj LBA4404. Bakterije su nosile binarni plazmid PBI121 ili njegove derivate s odsječcima promotora gena DC-8 iz mrkve. Rezultati su pokazali da starost i tip eksplantata utječe na uspješnost transformacije in vitro. Na eksplantatima 5 tjedana starih korjenčića uročnjaka nije bilo moguće potaknuti rast transgeničnog kalusa dok je to postignuto na eksplantatima 3 tjedna starih korjenčića. Također nije bilo moguće (iako se razvio transgenični kalus) potaknuti razvitak transgeničnih izdanaka na eksplantatima stabljike. Prilikom transformacije *in vitro* bilo je potrebno 15 do 16 tjedana od sjemenke do zrelog primarnog transformanta za razliku od *in planta* transformacije gdje je bilo potrebno 19 do 20 tjedana. U radu se diskutira o prednostima i nedostacima ove dvije metode transformacije.

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