GENETIC TRANSFORMATION OF MEDICAGO TRUNCATULA USING SYSTEM FOR DIRECT SOMATIC EMBRYOGENESIS PROMOTED BY TDZ

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ABSTRACT

The efficient procedure for Agrobacterium mediated transformation of Medicago truncatula cv. R1081 was developed. Using the advantage of recycling embryo formation of system for direct somatic embryogenesis promoted by TDZ, transgenic plantlets were obtained for a short period of 60 days. Transformation was performed with bacterial strain – LBA 4404, containing binary vector pBI 121 carries two genes - uid A gene expressing β-glucoronidase (GUS) and npt II gene for resistance to kanamicin. Different parameters of the gene transfer– pre-treatment of the embryo clusters, density of the bacterial suspension, time for inoculation and co-cultivation, type of selection were optimized. For evaluation of the transformation efficiency, histochemical GUS assay was performed and glucuronidase activity was detected in embryos, plantlets and seedlings of T1 progeny. Transgenic nature of plantlets was confirmed by npt II-specific PCR amplification and Southern hybridization. In the progeny transgenes segregated in Mendelian manner.

Introduction

The most common method for introducing DNA into Medicago genus - Agrobacterium tumefaciens system, is utilized extensively to introduce genes of agronomic importance and to study legume biology. Legumes are known for their calcitrant nature for plants regeneration and transformation. In the last years annual medic M. truncatula was proposed as a model sp., for molecular genetics studies because of its relatively small genome size and short life cycle. Recently, considerable effort has focused on an optimizing Agrobacterium tumefaciens mediated DNA transfer procedures. In general, this method is well worked out and transformation and regeneration in Medicago truncatula is routine now but application of procedures needs to be optimized for each genotype. During the last ten years transformation systems have been develop rapidly (1; 2; 3; 4; 5; 6). In order to avoid labor-intensive in vitro regeneration step) an in planta infiltration approach was established (7). It offers the possibility to easily produce large number of transformants but stable transgene expression in the progeny has to be confirmed.

Direct somatic embryogenesis is a preferable pathway for plant regeneration because due to the single cell origin of induced primary and secondary embryos in the most of the cases and avoiding callus phase, yields genetically stable plants.

The objective of this study was to establish an optimized procedure for Agrobacterium tumefaciens mediated transformation, on a system for direct somatic embryogenesis on solid media, which is fast and genotype independent.

1 Abbreviations: TDZ thidiazuron, BAP, 6-benzylaminopurine
Materials and Methods

Bacterial strain and plasmid
Transformation was carried out using: *A. tumefaciens* strain LBA 4404 carrying plasmid pBI 121, which contains two genes - *uid A*, expressing β-glucoronidase (GUS) activity and *npt II* (neomycin phosphotransferase) gene under the control of CaMV 35 S promoter and *nos* terminator.

Bacterial strain was maintained on agar (1.5%) solidified YEB nutrient medium (8), supplemented with 100mg/l Rifampicine (Rif) and 50mg/l Kanamycine (Km). Bacterial suspensions were incubated in the same liquid medium at agitation 250 rpm for 24h at 28 ºC prior to inoculation of plant explants. For inoculations bacterial suspensions at density OD600 = 0.3; 0.7; 0.9 were used.

Plant material, culture media and conditions

**Plant material**: The experiments were carried out with *M. truncatula* cv. R 108-1. The seeds were sterilised with 70% ethanol for 30 s, 0.2 % HgCl2 solution for 3-4 min and rinsed five time with sterile distilled water.

**Explant preparation**
As explants were used clusters of embryos developed from cotyledonary and petiole base after 20-25 day on solid medium supplemented with TDZ for direct somatic embryogenesis previously described in (9).

**Culture media**
1. In vitro cultivation: For seeds germination and maintenance of donor plants hormone-free MS medium (10) was used.
2. Medium for plant regeneration contained MS salts, Morel vit., 3% sucrose, 0.25% Phytogel and supplemented with 0.5 mg/l TDZ (9). Growth regulator were added after filter sterilisation (0.22µ Millipore).
3. Conversion to plants: The elongation of regenerated clusters of transgenic embryos were performed on MS1 solid medium supplemented with 0.05 mg/l BAP, 250 mg/l casein hydrolisate (11) and 50 mg/l Km (Kanamycine). Regenerated plantlets were rooted and maintained on selective MS (50 mg/l Km), basal medium. All regenerants, capable to develop root system under the selective pressure were cloned, part of them analyzed and transferred to soil and grown at the greenhouse condition.

**Genetic transformation**
Clusters were removed from the initial explants (Fig. 1a) and transfer on fresh regenerative medium containing 0.5 mg/l TDZ for 24 hours pre-cultivation. Embryo clusters were submerged into the over night bacterial suspension and cut by razor blade on very small peaces (1-2 mm). The inoculation extended for 10, 20 and 30 min. After 24 hours of co-cultivation peaces from embryo clusters were washed 3 times with sterile dH2O, soaked on filter paper and transferred to the selective regeneration medium, containing – 50 mg/l Km and 400 mg/l Claforan (Clf) for removing of the bacteria.

Putative transgenic shoots were isolated after 60 days of cultivation on selective MS medium containing 50mg/l Km.

**Killing curve and selection of transgenic structures**
For determination of experimental dose, the selective agent - Kanamycin (Km), in concentrations 25, 50, 75 and 100mg/l were examined. A non-interrupted system for selection was applied.

All plant material was cultivated in growth chambers at 26º C, 70% humidity; 16/8 h photoperiod and 2500-3000 lux light intensity.

**β-glucoronidase activity assay**
The histochemical GUS assays were based on method described by Jefferson et al. (12) with minor modifications. For histochemical staining, clusters of embryos or organs of already regenerated plantlets (plant root or leaves) were incubated overnight at 37º C in 0.5 mg/ml X-gluc solution.
Fig. 1. Explants for transformation and GUS analysis in *Medicago truncatula* R 108 1. a - upper-cotyledonary base with clusters of embryos formed 20 days on induction medium.; middle-recycling embryo formation from cut clusters after transformation; down-negative control on selective medium; b - GUS positive embryo; e - Rooted GUS positive embryo; d - Histological section of GUS positive embryo; e - Histological section of leaf of transformed plant.

(5 mg 5-bromo-4-chloro-3-indolyl -B- D-glucuronide) dissolved in 50 µl formamide and 100 mM sodium phosphate buffer, pH 7.0, 0.1% (v/v) Triton X-100, 20% (v/v), methanol. To prepare tissue for histological analysis, after GUS staining, *M. truncatula* leaf tissue and clusters of somatic embryos were collected and fixed using 4% paraformaldehyde supplemented with 0.25% glutaraldehyde in 0.01M phosphate buffer at 4ºC overnight. The tissue was processed through two series of ethanol series and xylene and embedded in paraplast (Sigma). Sections (20µm) were cut from the embedded tissue. Wax was removed by a brief incubation in xylene. Photographs were taken by dark-field illumination on a Labophot-2 Microscope (Nikon) using Kodak Ektachome 64T film.

**Molecular analysis of the putative transgenic plants.**

PCR detection for *npt* II gene

Genomic DNA from transgenic plants was isolated according modified method of (13). PCR was performed in a volume of 25 µl consisting of 1x PCR buffer, the four deoxyribonucleotides (200 µM each), two relevant primers (5’- GTG GAG AGG CTA TTC GGC TA - 3’; 5’-CCA CCA TGA TAT TCG GCA AG-3’) 15 µM each, 0.5 units of Taq DNA polymerase and 200ng of the extracted DNA. The mixture was primarily denaturated at 94ºC for 3 min, and 35 cycles of 30 sec at 94ºC, 45 sec at 55 ºC and 45 sec at 72ºC. The reaction was completed by elongation step at 72ºC for 5 minute. An aliquot (routinely 10 µl) of the reaction mixture was used for analysis of the amplified products by agarose gel electrophoresis.
Southern blot analysis

PCR-amplified DNA was subjected to electrophoresis through a 0.8% agarose gel. DNA was denatured by an alkaline treatment and transferred to Hybond-N (Amersham) nylon membrane by capillary blotting as described by (13). For labeling and detection, the ECL direct nucleic acid system was used (ECL Random Prime System, AMERSHAM).

Results and Discussion

Once developed, embryo clusters (Fig. 1a upper) were transferred on fresh regenerative medium and used as initial explants for transformation. Wounding of clusters by razor blade caused necrosis of small explants pieces but 7 days after transformation on selective regenerative medium the recycling clusters of embryos appeared (Fig 1a middle) in contrast to the non-transformed control (Fig 1a down).

Pre-cultivation of the clusters (Fig. 1a) for 24h on fresh 0.5 mg/l TDZ induction medium, prior to Agrobacterium-mediated transformation increased percentage of newly regenerated clusters (up to 65) after transformation.

It was established that the optimal density of the bacterial suspension was OD600 = 0.7. Our observations showed that lower than above bacterial density caused very slight or absence of any bacterial infection after co-cultivation. On the contrary, the concentrations higher than the optimal one led to strong contamination of the plant material even in the next passages on fresh medium. The bacterial re-infection decreased the regeneration capability of the cut clusters and they became necrotic and died. Time for inoculation of 20 minutes and time for co-cultivation of 24 hours on regeneration medium were determined as the most effective. Long term co-cultivation (48 h) leaded to higher development of bacterial infection and further elimination of bacteria was impossible.

The determination of killing curve for the selective agent and a proper scheme for selection of putative transgenic tissue is also critical step. Embryo clusters and intact plants from M. truncatula cv. R 1081 were tested for their capability for recycling embryo formation and rooting at different concentrations of Km in the media. De novo clusters formation was not observed at concentration higher then 50mg/l Km. The higher concentrations of Km negatively influenced on the processes of regeneration even of the transformed cells. At concentration higher then 50 mg/l Km the regenerated intact plants stayed green, but formation of roots was not observed.

Forty putative transgenic rooting embryos or plantlets were selected randomly for histochemical and histological GUS assay in order to confirm their glucoridas activity. All of them showed positive GUS activity - whole surface of embryos and rooting embryos, leaf or root were colored in blue. Among them 11 randomly chosen plants were selected for analyzing their neomycin phosphotransferase activity. 9 out of 11 tested plants showed amplification of the same fragment (Fig. 2). All amplicons were visible on ethidium-bromide-stained gel. In order to confirm the integration of npt II gene and also to prove the results from PCR analysis, Southern blot hybridisation of the PCR products of 7 plantlets was performed. All PCR products were identified as npt II amplification products. The performed analysis confirmed that the fragments were not result from non-specific amplification (Fig. 3, Table).

The confirmed transformants possessed normal phenotype and were successfully transferred to greenhouse conditions. Medicago truncatula transgenic plants were fertile and produce seeds after 4 – 5 months. The inheritance of the transgene (npt II ) was studied by germination of seeds collected from three randomly chosen T₀ plants, on MS medium containing 100 mg/l Km. 52 of seeds showed normal
Fig. 2. Gel electrophoresis of PCR amplified products (550 bp npt II fragments); “+c”-positive control; “-c”-negative control; lanes 1-11 tested clones; lanes 3, 5 putative transformed clones, which do not amplified npt II fragment.

Fig. 3. DNA hybridization of PCR products obtained after npt II amplification; lanes 1-7 positive tested clones; “-c” negative control; “+c” positive control.

**TABLE**

<table>
<thead>
<tr>
<th>Number of transformed clusters</th>
<th>Number of recycled clusters Km positive</th>
<th>GUS positive plantlets</th>
<th>PCR positive</th>
<th>npt II positive</th>
<th>TE %</th>
</tr>
</thead>
<tbody>
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<td>100</td>
<td>65</td>
<td>37</td>
<td>9</td>
<td>7</td>
<td>9</td>
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TE - transformation efficiency in %.

growth and 20 filed to developed. Inheritance of npt II gene indicated segregation ratio in Mendelian manner (3:1). These T₁ seedlings analyzed further by histochemical staining for GUS activity showed the inheritance of reporter gene and the expression pattern typical for 35 S promoter – blue color detected in different plant parts (Fig. 4 a,b).

The previously reported from us procedure for direct somatic embryogenesis on solid medium (9) is a fast and genotype independent, leading to embryo formation from explants with a small meristematic zone like a cotyledonal and petiole base. Take the advantage of recycling embryo formation we succeeded to obtain transgenic plantlets – GUS positive and Km resistant for a short period of time (55 –60 days) compared to the other protocols for transformation of *Medicago truncatula* (2, 6). All protocols for transformation of this species published up to now were via indirect somatic embryogenesis and took a long period for the production of transgenic plants.
Fig. 4. GUS analysis of T1 seedlings. a - stem and petiole tissue showing gus activity; b - leaf tissue showing gus activity.

The established pre-cultivation period of embryo clusters on fresh regeneration medium 24 h, prior to Agrobacterium-mediated transformation increased the transformation frequency from 50 (non pre-treated clusters) to 65%. Pretreatment with TDZ is one of the key factors, which acts as a mitotic trigger and re-activates cell division (14). The number of dividing cells increased and influenced positively the transformation frequency.

The early started (after co-cultivation) non-interrupted selection was chosen as an optimal at the conditions of our experiments. The application of early selective pressure to the cut on small pieces embryogenic clusters, allowed to be avoided the competition of non-transformed cells immediately after co-cultivation. The selective medium, containing 50mg/l Km did not inhibit the formation of newly developed embryo clusters and further rooting of the putative transformants. The observed GUS staining pattern (Fig. 1 b, c) was typical for this gene under the control of the constitutive 35 S promoter. The histological observation of GUS pattern viewed at low magnification of thin section (Fig. 1 d, e) confirmed uniform staining in cells throughout the embryo surface and the leaf tissue.

The presence of non transgenic regenerants, survived under the selective pressure could be explained with the fact that Km is not enough strong selective agent (15), but also with the absence of direct contact of each somatic embryo from clusters with the selection medium. The obtained transformation efficiency (Tab.1) was calculated on the base of PCR npt II positive plants, because plants for Southern hybridization were selected randomly from the PCR positive. The results confirmed that transformation efficiency in this study was compatible with the other authors (3; 6; 16).

The increased concentration of selective agent for seedlings was used for the proper selection efficiency. This high antibiotic concentration eliminates development of non-transformed seedlings and confirmed the Mendelian manner of inheritance of the transgene.

Conclusions

As a result of our experimental work, transgenic Medicago truncatula cv. R 108 1 plants, containing genes for resistance to Km and GUS reporter gene were obtained on the base of a system for direct somatic embryogenesis on solid medium. The time for obtaining transformed plants was shortened at compatible transformation efficiency. This is very important for model species like Medicago truncatula.

REFERENCES

8. Van Larebeke N., Genetello C., Hernalsteens J.,