Chapter 18

Transgenic *Hypericum perforatum*

G. Franklin, Margarida M. Oliveira, and Alberto C.P. Dias

Summary

Plant transformation is an important tool with many applications in modern plant biology. Although this technique is primarily used to produce superior crop varieties, it is also being utilized to answer basic questions concerning gene function and regulation in contemporary functional genomics research. In our laboratory, we have established a transformation system for *Hypericum perforatum*. This protocol involves the transfer of foreign DNA into *H. perforatum* organogenic nodule explants *via* particle-bombardment and the regeneration of shoots from the explants under selection pressure. We have successfully used this method to express ß-glucuronidase and hygromycin phosphotransferase genes in *H. perforatum*. Molecular analyses of putative phenotypically normal transgenic plants show stable integration of the transgenes into the plant nuclear genome. Here we describe the procedure for the transformation of *H. perforatum*.

Key words: *Hypericum perforatum*, Organogenic nodular suspension culture, GUS gene, HPT gene, Particle-bombardment, Transgenic plant, Polymerase chain reaction analysis, Southern blot analysis

1. Introduction

Hypericum perforatum L. (St. John's Wort) is an important medicinal plant that has been used since ancient times for the treatment of numerous ailments. Recent clinical studies demonstrate that *H. perforatum* extracts are efficient in the treatment of mild to moderate depression *(1, 2)*. The extract is also reported to possess antiviral *(3)*, anticancer *(4)*, neuroprotective *(5)* and antioxidant *(6)* properties. The pharmaceutical importance of *H. perforatum* extract (secondary metabolites) is the main driving force behind the research that is focused on HP cell cultures *(7–11)*. However, the cell and tissue cultures for large-scale production of secondary metabolites has so far

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achieved only limited success because of the low and unreliable yield of the products. Although significant improvements in product yields have been achieved through conventional biochemical approaches and manipulation of culture and process factors, the reproducibility of results is still a matter of concern *(12)*.

Biosynthesis of therapeutically useful compounds can be effectively improved in medicinal plants by altering the expression of transcription factors or structural genes through metabolic engineering *(13–15)*. As the pharmacologic activities of *H. perforatum* extract are largely attributed to compounds like hypericin and hyperforin that are exclusively produced in this species, improving their production is an important target for genetic manipulation. In spite of the availability of excellent regeneration protocols *(16–19)*, this goal is not realized satisfactorily so far because of the poor knowledge about the biosynthetic pathways involved and also because of the absence of a suitable genetic transformation system for the species.

Particle-bombardment (biolistics) is a versatile technique, by which very different cell types can be transformed *(20, 21)*. Because this technique makes use of physical processes to accelerate DNA directly into intact tissues, it has the advantage of avoiding plant cell defense responses, frequently observed in recalcitrant plants against *A. tumefaciens(22, 23)*. Hence, this technique has been successfully used in the genetic transformation of a wide variety of plant species that remain otherwise recalcitrant to *Agrobacterium*-mediated transformation *(24–26)*. Moreover, this technology can deliver large number of genes into the target cells in a single step *(27)*, which is often necessary for the manipulation of metabolic pathways of plants *(28)*.

Because *H. perforatum* remains highly recalcitrant to *A. tumefaciens* mediated transformation *(22)*, particle-bombardment would be an extremely useful alternative technology in efforts to improve this medicinal plant. Hence, we have developed a particle-bombardment protocol for genetic transformation of *H. perforatum*, which can be applied in the genetic improvement programs of this important medicinal plant. So far, we have used this procedure to introduce the ß-glucuronidase (GUS) gene *(29)* and stilbene synthase gene (G. Franklin and A.C.P. Dias, unpublished data) into the *H. perforatum* nuclear genome.

2. Materials

2.1. Plant Material

H. perforatum seeds are available from many commercial sources including Richters Seeds (Goodwood; ON, Canada). The target tissues **(Fig. 1)** for particle-bombardment mediated

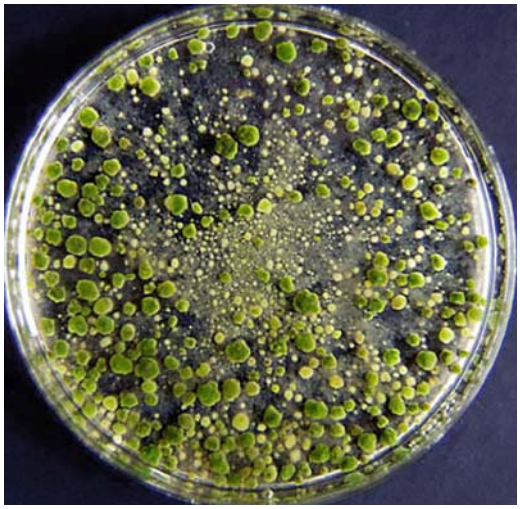

Fig. 1. Organogenic cell suspension of *H. perforatum* variety 'Helos' showing ONS explants.

transformation of *H. perforatum* can be obtained as described in **Subheading 3.4**.

2.2. Plasmid Vector

The binary vector used in the present study (pCAMBIA1301, Cambia, Australia) harbors the hygromycin phosphotransferase (HPT) gene as the selectable marker and the GUS gene disrupted by a catalase intron as reporter gene. Both genes are driven by the CaMV 35S promoter and are cloned in opposite orientation **(Fig. 2)**. Plant expression vectors with many other combinations of marker and reporter genes are also available from Cambia and from other sources. We use *E. coli* strain DH5 alpha (Invitrogen, USA) for plasmid multiplication and Wizard® plus midipreps DNA purification system (Promega, USA) for plasmid isolation. Plasmid DNA should be stored at –20°C.

2.3. Plant Culture Media and Components

Plant culture media used in the present study are based on Murashige and Skoog (MS) *(30)* formulation.

1. 10X Murashige and Skoog (MS) basal salt mixture with and without vitamins (Duchefa Biochemie, The Netherlands).
2. 1000X MS vitamin cocktail: Dissolve 200 mg glycine, 50 mg nicotinic acid, 50 mg pyridoxine–HCl and 1 g thiamine–HCl in 100 mL of deionized water.
3. Other additives: myo-inositol, D-Mannitol (Sigma, St. Louis, MO), D-Sorbitol (Sigma), Sucrose (Panreac, Spain) and agar (VWR, Belgium)

2.3.1. Plant Growth Regulator Stock Solutions

1. Plant growth regulators (PGRs) including 6-benzylaminopurine/N6-benzyladenine (BA), Indole-3-butyric acid (IBA), α-napthaleneacetic acid (NAA), and Kinetin (furfurylaminopurine) (Sigma).

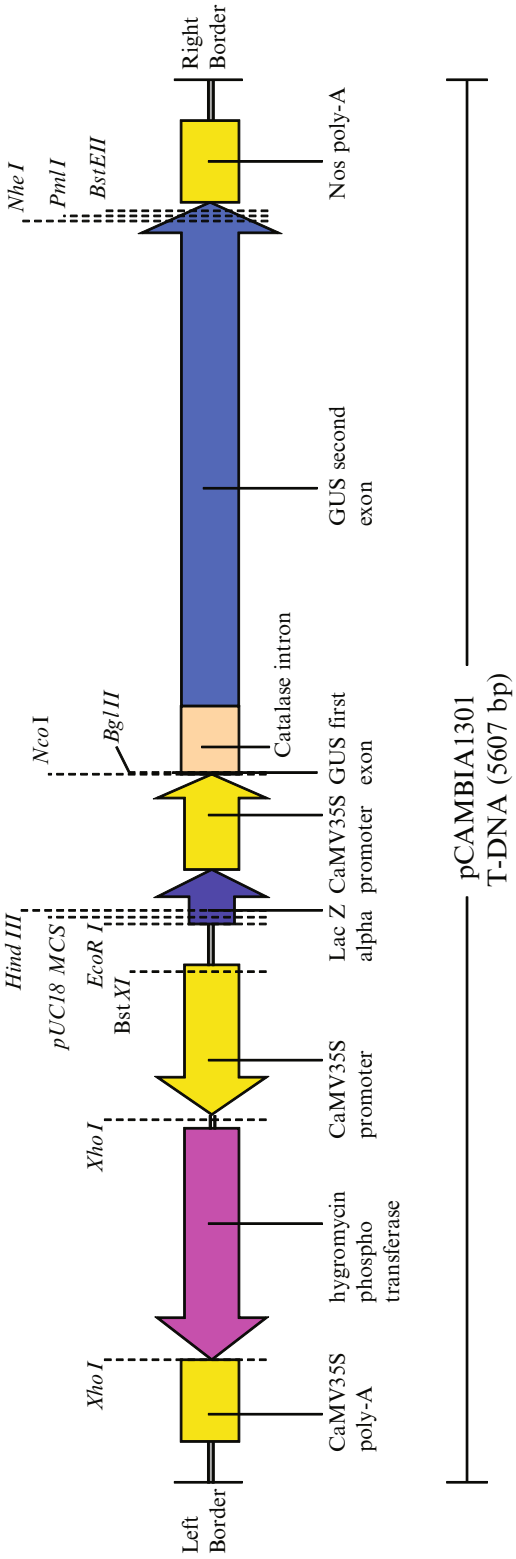

Fig. 2. T-DNA of pCAMBIA1301 showing restriction sites.

2. Stock solutions of the above PGRs can be made by dissolving the required amount in a minimal volume of 1 N NaOH and diluting with distilled water to obtain 1 mg/mL concentration and stored at 4°C.

2.3.2. Antibiotic Stocks

Antibiotics are available from several commercial sources. We have used hygromycin B and ticarcillin clavulanate (timentin) (Duchefa) and kanamycin sulphate (Calbiochem, USA).

1. Prepare stock solutions of desired antibiotic concentrations by dissolving them in distilled water. We generally prepare 10- , 50 and 250-mg/mL stock solutions respectively for hygromycin, kanamycin, and timentin.
2. Filter sterilize kanamycin and timentin using 0.2–0.45 μm pore size syringe filters (Sarstedt, Numbrecht, Germany). Because of its high toxicity, hygromycin B does not need sterilization.
3. Store all antibiotic stocks at –20°C as frozen 1-mL aliquots.

2.4. Particle Bombardment

Particle Delivery System PDS-1000/He, various sizes of microcarrier gold particles, macrocarriers, macrocarrier holders, stopping screens and rupture discs including 1100 psi (Bio-Rad, USA).

1. Maintain and multiply plasmid of interest (pCAMBIA1301) as described in **Subheading 2.2.**
2. Prepare 0.1 M stock solution of spermidine-free base (Sigma) immediately after opening the bottle and store as 100 μL aliquots at –20°C.
3. Prepare fresh $CaCl_2$ solution (2.5 M).

2.5. Analysis of Transgenic Plants

2.5.1. GUS Solution

1. Dissolve 100 mg 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-GlcA) (Sigma) in 1 mL *N, N*-dimethylformamide or dimethylsulfoxide (Sigma).
2. Add 15 mL 100 mM NaH_2PO_4 buffer, pH 7.0; 2.0 mL 0.5 M ethylenediamine tetraacetic acid (EDTA); and 10 mL 1% *(v/v)* Triton-X 100.
3. Make up the final volume to 100 mL with sterile distilled water.
4. Store the solution as desired aliquots at –20°C.

2.5.2. Polymerase Chain Reaction

1. Isolate genomic DNA from putative transgenic plants. We use DNeasy plant mini kit (Qiagen, Germany).
2. Components for polymerase chain reaction (PCR): gene-specific primers: 5X Taq DNA polymerase, 10 mM dNTP mix, and 25 mM $MgCl_2$ solution.
3. Dissolve primers in distilled water to a final concentration of 5 pmol/μL. We use the following primer sequences to amplify GUS and HPT genes.

   GUS gene forward – 5′GATCGCGAAAACTGTGGAAT3′

   GUS gene reverse – 5′TGAGCGTCGCAGAACATTAC3′

HPT gene forward – 5′ATTTGTGTACGCCCGACAGT3′

HPT gene reverse – 5′GGATATGTCCTGCGGGTAAA3′

4. Store all the components at –20°C until use.
5. Perform PCR analysis of genomic DNA in a thermocycler (Mastercycler gradient®, Eppendorf, Germany).

2.5.3. Southern Blot Analysis

1. Restriction endonuclease *Eco*RI (Fermentas, USA).
2. Hybond nylon membrane (Amersham Biosciences, UK).
3. Ultraviolet (UV) cross-linker (Stratagene, USA).
4. Church buffer: 250-mM sodium phosphate buffer, pH 7.2; 1% *(w/v)* bovine serum albumin (BSA); 7% *(w/v)* sodium dodecyl sulfater (SDS); and 1 mM EDTA.
5. α-[^{32}P] dCTP (Amersham), Prime-a-Gene® labeling kit (Promega).
6. Hybridization oven/shaker (Amersham) and phosphorimager (Bio-Rad).

3. Methods

3.1. Media Preparation

Refer to **Table 1** for the media components.

1. For all media, adjust the pH to 5.8 before autoclaving at 121°C for 15 min.
2. Add antibiotics, if needed, after cooling the media to 50–60°C.
3. Pour 25 mL solid medium in each sterile plastic Petri dish and solidify in a flow hood.

3.2. Sterilization of Instruments

1. Sterilize instruments for explant preparation (forceps, scalpel etc.) by dipping them in 90% *(v/v)* ethanol and flaming. It is important to cool them before use.
2. Alternative methods of sterilization are also possible.

3.3. Seed Germination

1. Take approximately 50–100 *H. perforatum* seeds in an Eppendorf tube containing 1 mL sterile water and add one drop of Tween-20. Keep in dark at 4°C.
2. After 12 h, discard the solution. Decontaminate the seeds with 70% *(v/v)* ethyl alcohol for 60 s and with commercial bleach containing 1.5% *(v/v)* active chlorine for 3 min (*see* **Note 1**).
3. Wash the seeds three times in sterilized distilled water and blot-dry on a sterile filter paper.

Table 1
Media Composition

Component	WA	½ MS	MSB	CIM	ONI	REG	OSM	SEL	RT
MS basal salts (g/L)		2.15	4.3	4.3	4.3	4.3	4.3	4.3	2.15
Sucrose (g/L)		15.00	30.0	30.0	30.0	30.0	30.0	30.0	15.00
Vitamins									
Myo-inositol (mg/L)		50	100	100	100	100	100	100	50
MS vitamins (mL/L)		0.5	1	1	1	1	1	1	0.5
Agar (g/1 L)	8	8	8	8		8		8	8
PGRs									
Kinetin (mg/L)				0.5					
NAA (mg/L)				1	0.5	0.1	0.1	0.1	
BAP (mg/L)						0.1	0.1	0.1	
IBA (mg/L)									0.5
Antibiotics									
Timentin (mg/L)								500	500
Hygromycin (mg/L)								20	20
Osmoticum									
D-Mannitol (g/L)							34		
D-Sorbitol (g/L)							34		

4. Transfer the disinfected seeds onto WA (water–agar) medium for germination.
5. After 10–15 d, transplant the seedlings into Baby Food Jars containing 50 mL ½MS (half strength MS basal) medium for further growth (*see* **Note 2**).

3.4. Establishment of Organogenic Nodular Cell Suspension Culture

1. Transfer the *H. perforatum* seedlings aseptically to a sterile Petri dish containing sterile distilled water (*see* **Note 2**).
2. Excise the leaves and transfer them to CIM (callus induction medium). Green compact callus induction can be seen in 15 d (*see* **Note 3**).
3. Cut the green compact calluses into pieces and transfer to organogenic nodule induction (ONI) medium. Keep the flasks on a rotary shaker at 120 rpm.
4. Organogenic nodules **(Fig. 1)** generally appear in the culture after 4–5 subcultures (*see* **Note 4**).

5. Collect these nodules using steel mesh screen (# 40, Sigma) and culture separately (*see* **Note 5**).
6. This organogenic nodular suspension (ONS) culture can be maintained for many years by subculturing a 10-mL aliquot to 70-mL ONI medium every month (*see* **Note 6**).

3.5. Pretreatment of Organogenic Nodules for Bombardment

1. Harvest ONS from the cultures using a sterile steel mesh screen (# 40, Sigma) and transfer them to OSM (osmotic medium) for osmotic treatment. This treatment should not exceed 4 h (*see* **Note 7**).
2. Transfer 1-mL ONS along with OSM to the centre of a sterile round Whatman No1 filter paper disk (*see* **Note 8**).
3. Following the absorption of the excess OSM, carefully place the paper disc along with the ONS tissues in plastic Petri dishes containing 25-mL solidified OSM.
4. Plates are now ready for bombardment.

3.6. Plasmid DNA Multiplication and Isolation

1. Transform chemically competent *E. coli* with pCAMBIA1301 following the manufacturer's protocol.
2. Transformed *E. coli* can be maintained as glycerol stocks. When necessary, initiate 100 mL broth culture in Luria bertani (LB) medium augmented with 50 mg/L kanamycin.
3. Isolate plasmid DNA from bacterial culture grown overnight (*see* **Note 9**). There are several protocols available for plasmid isolation from *E. coli*. We use Wizard®plus midipreps DNA purification system following manufacturer's instructions.
4. Quantify the plasmid DNA in a spectrophotometer and adjust to 1 μg/μL using TE (*see* **Note 10**) and store at –20°C.

3.7. Preparation of Gold Particles

1. Weigh out 20 mg gold particles, 1 μm size, in a sterile 1.5-mL Eppendorf tube, add 1 mL 70% *(v/v)* ethanol and vortex vigorously for 5 min.
2. Allow the gold particles to settle down by resting the tube for 15 min.
3. Pellet the settled gold particles by spinning for 5 s in a microfuge and discard the supernatant.
4. Add 1 mL sterile distilled water to the pellet and vortex for 1 min.
5. Allow the particles to settle down by resting the tube for 5 min.
6. Pellet the particles by spinning for 5 s in a microfuge and discard the supernatant.
7. Repeat **steps 4–6** two more times.
8. Suspend the gold particles in 350 μL 50% *(v/v)* glycerol and store at 4°C (*see* **Note 11**) until use.

3.8. Coating Gold Particles with Plasmid DNA

1. Pipet out 87.5 µL that is equivalent to 5 mg gold particles from the glycerol stock to a sterile 1.5-mL Eppendorf tube and vortex vigorously.
2. While vortexing, add 5 µL 1 µg/µL plasmid DNA, 87.5 µL 2.5 M $CaCl_2$ and 35 µL 100 mM spermidine sequentially into the tube (*see* **Note 12**).
3. Rest the mixture on ice for 10 min.
4. Centrifuge for 10 s in a microfuge to pellet the DNA coated gold particles and discard the supernatant.
5. Gently resuspend the pellet in 100 µL 100% ethanol and keep on ice (*see* **Note 13**).

3.9. Particle Bombardment

1. Set up the PDS-1000/He Particle Delivery System in a flow hood and arrange the helium gas cylinder and the vacuum pump conveniently to connect with it (*see* **Note 14**). For an interactive guide, refer the manufacturer's website http://www.bio-rad.com
2. Wipe the target shelf, macrocarrier launch assembly and the particle delivery chamber of the apparatus with absolute ethanol.
3. Immerse the macrocarrier holders, macrocarriers, and stopping screens in absolute ethanol and keep rupture discs immersed in isopropanol (see Note 15).
4. Unscrew the rupture disk retaining cap from the gas acceleration tube and place rupture disk (1100 psi) in the recess of the cap.
5. Take the macrocarriers out of ethanol and place on a sterile Whatman no. 1 filter paper to allow ethanol to evaporate and position them into the macrocarrier holders.
6. Briefly vortex the DNA precipitated gold particles for 5 s to disperse any clumps and pipet out 10 µL onto the centre of the each macrocarrier, spread evenly and air-dry (*see* **Note 16**).
7. Arrange a macrocarrier holder containing dry macrocarrier coated with microcarrier particles in the launch assembly in such a way that the microcarriers face the target desk.
8. Place the osmotic plates containing tissues 9 cm below the stopping screen on the target desk (*see* **Note 17**).
9. Close and evacuate the bombardment chamber to 28" of mercury (*see* **Note 18**).
10. Fire the microcarriers onto the target tissue.
11. Perform **steps 4–11** for each plate.
12. Seal the plates containing bombarded tissues with parafilm and incubate in darkness.

3.10. Regeneration of Transgenic Plants

1. Transfer ONS tissues aseptically to REG (regeneration) medium 4 h after bombardment and incubate in dark (*see* **Note 19**).
2. After 2 d, transfer the tissues to selection medium (*see* **Note 20**).
3. Maintain all the cultures in dark until the formation of callus **(Fig. 3a)** or shoot initials (*see* **Note 21**).
4. Transfer ONS with calluses and/or shoot initials **(Fig. 3b)** to MSB medium containing 20 mg/L hygromycin for shoot elongation (*see* **Note 22**).
5. Once the shoots are reached 3- to 5-cm height **(Fig. 3c)** excise them from the explant and transfer to Baby Food Jars containing 50 mL of rooting medium.
6. Rooting can be observed from hygromycin-resistant shoots within 25 d **(Fig. 3d)**, while nontransformed (hygromycin-susceptible) shoots will be completely killed **(Fig. 3d**, *arrows***)**.
7. Fill the jars containing rooted plants with sterile distilled water and leave at room temperature for 2 d (*see* **Note 23**).
8. Remove the plants from the medium without damaging the root system and wash thoroughly in running tap water to remove the traces of rooting medium (*see* **Note 24**).
9. Transfer the plants to plant propagation system **(Fig. 4a)** and cover with polyethylene bags to maintain humidity.
10. Acclimatize the plants by gradually reducing the humidity by making holes in the polyethylene bags and by gradually exposing the plants to sunlight for a period of 1 wk.
11. Transfer the hardened plants to garden pots **(Fig. 4b)** containing soil:compost (1:1), irrigate regularly with tap water and grow under controlled environment. We use growth chamber with 25 µmol/m^2 s 16 h/d incident radiation, 70% humidity and 26°C temperature (*see* **Note 25**).

3.11. Analyses of Transgenic Plants

3.11.1. Enzymatic Histochemical GUS Assay

1. Thaw the frozen aliquots of GUS solution to room temperature.
2. Thoroughly wash the tissue samples in distilled water and put them into tubes containing GUS solution (*see* **Note 26**).
3. Cover the tubes with aluminium foil and incubate at 37°C for 12–24 h.
4. Remove chlorophyll from the tissues with 70% *(v/v)* methanol/ethanol to visualize the result more clearly.
5. Characteristic deep blue coloration is the indication of GUS gene expression in the tissues **(Fig. 5)**.

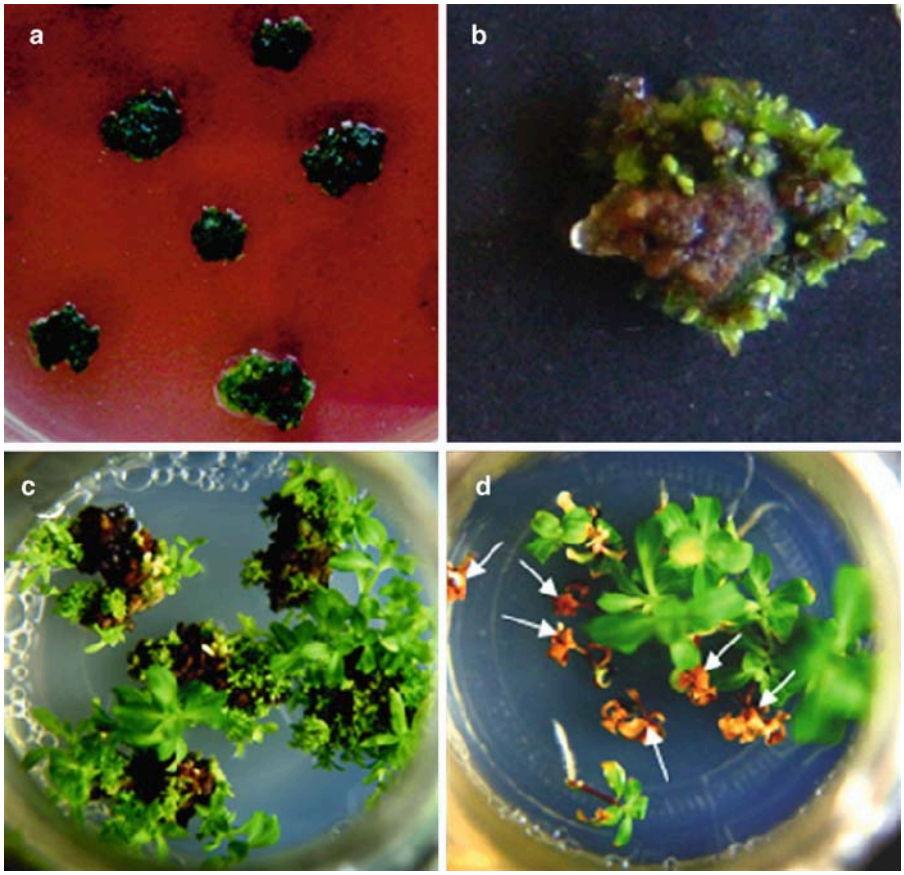

Fig. 3. Regeneration of shoots from ONS explants bombarded with pCAMBIA1301. (**a**) Callus development from bombarded explants after 10 wk on selection medium. (**b**) Shoot regeneration from hygromycin-resistant callus. (**c**) Cultures with uniform green shoots on selection medium. (**d**) Regenerated shoots on rooting medium containing hygromycin showing the death susceptible shoots (*arrows*) and root initiation from resistant shoots.

3.11.2. Polymerase Chain Reaction Analysis

1. Take 50 ng DNA from each sample including the putative transgenic plants, pCAMBIA1301 (positive control) and non-transformed plant (negative control) into sterile PCR tubes. Make up the volume to 10 µL with PCR grade water.
2. Prepare a master mix cocktail sufficient for the desired number of reactions, each with 2 µL forward primer, 2 µL reverse primer, 0.5 µL dNTP mix, 5 µL PCR buffer, 2 µL $MgCl_2$, 0.25 µL Taq DNA polymerase and enough water to bring the volume to 15 µL. Distribute 15 µL to each PCR tubes containing DNA. Mix well by pipetting up and down gently.

Fig. 4. Hardening of transgenic plants. (**a**) Establishment of rooted plants in JIFFY 7 plant propagation system (Lisbon, Portugal). (**b**) A transgenic plant growing in garden pot after hardening.

3. Amplify the specific fragments of transgenes with a hot start at 94°C for 4 min, followed by 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 2 min) and extension (72°C, 2 min), with a final extension of 10 min at 72°C in the thermocycler.
4. Resolve the PCR products in 0.8% *(w/v)* agarose gel electrophoresis. The expected fragment sizes are 1.3 kb for GUS gene and 0.8 kb for HPT gene **(Fig. 6)**.

3.11.3. Southern Blot Hybridization Analysis

1. Digest 20 μg genomic DNA from control and PCR positive plants with *Eco*RI following the manufacturer instructions.
2. Load the restriction-digested DNA and proper molecular weight marker in 1% *(w/v)* agarose gel and run at 30 V overnight (*see* **Note 27**).

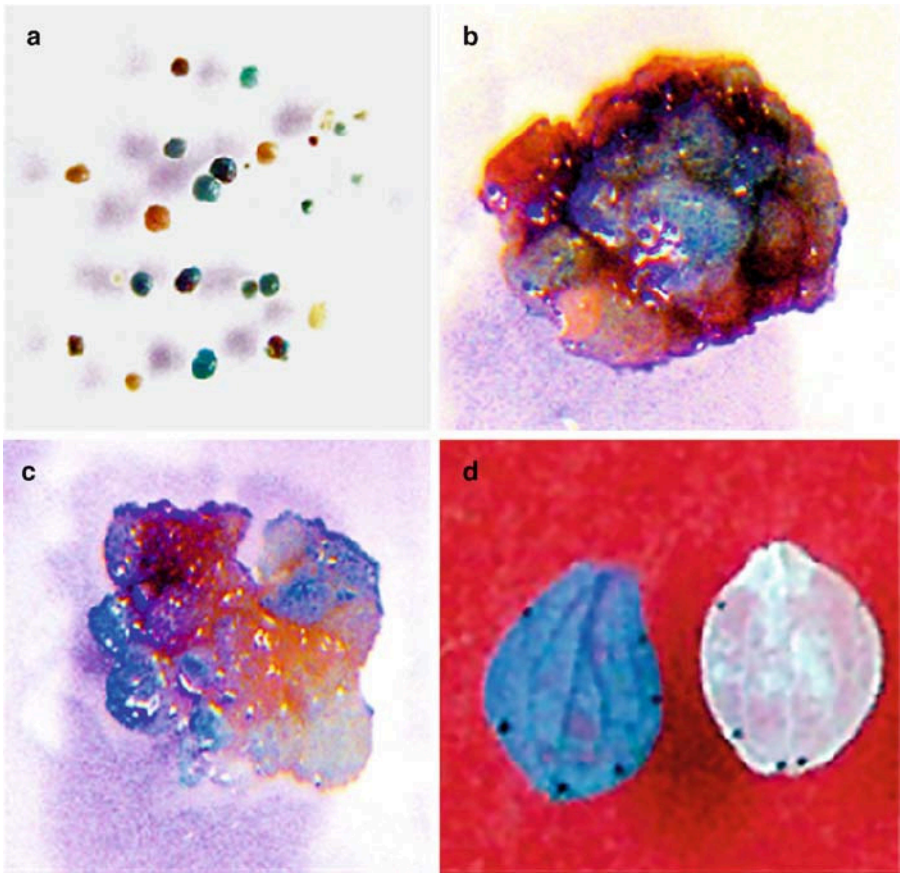

Fig. 5. Expression of GUS gene during the development of transgenic *H. perforatum*. (**a**) ONS explants bombarded with pCAMBIA1301 DNA using 1100 psi rupture disk with 9.0 cm flying distance showing many explants with transient GUS expression and others with no expression. (**b**) Close up view of an explant 10 d after bombardment showing several GUS foci indicating stable transgene expression. (**c**) ONS explant showing GUS activity in the newly formed calluses. (**d**) Leaf of a transgenic *H. perforatum* plant showing GUS activity (control leaf in the right).

3. Transfer the electrophoresed DNA onto a nylon membrane by alkaline capillary blotting.
4. Crosslink the DNA to the membrane using UV Stratalinker 1800, Stratagene under autocrosslink mode (*see* **Note 28**).
5. Prehybridize the membrane for 3 h in church buffer at 55°C.
6. Label a GUS gene specific fragment with α-[^{32}P] dCTP and transfer to the hybridization solution.
7. After 16 h hybridization at 55°C, remove the solution. Wash the membrane at 55°C twice with 2X SSC + 0.1% *(w/v)* SDS (each for 15 min) and with 0.1X SSC + 0.1% *(w/v)* SDS for 5 min.

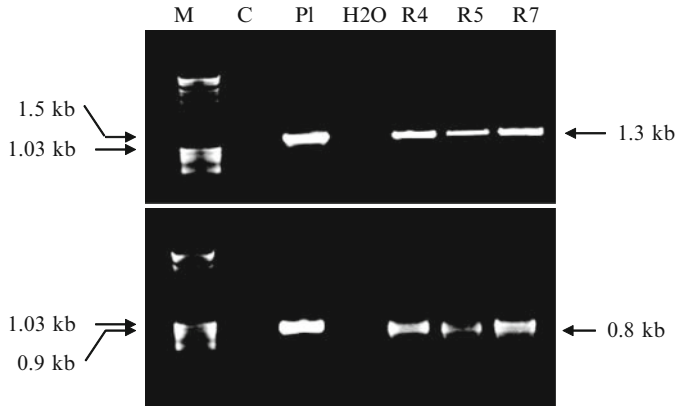

Fig. 6. PCR analyses of transgenic plants. (**a**) Agarose gel electrophorogram of GUS gene PCR amplification products. *Lanes*: *M* Molecular size marker (MassRuler, Fermentas), *C* control plant, *Pl* plasmid pCAMBIA1301 positive control, *H2O* water control, *R4, R5* and *R7* are plants positive in the GUS assay showing the amplification of expected 1.3-kb fragment of GUS gene. (**b**) Agarose gel electrophorogram of HPT gene PCR amplification products. *Lanes*: *M* Molecular size marker (MassRuler, Fermentas), *C* control plant, *Pl* plasmid pCAMBIA1301 positive control, *H2O* water control, *R4, R5* and *R7* are hygromycin resistant plants showing the amplification of the expected 0.8 kb fragment of HPT gene.

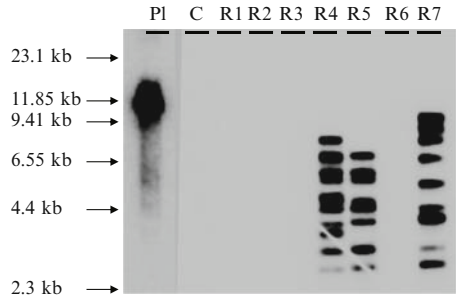

Fig. 7. Southern blot analysis of transgenic plants. Southern blot analysis of DNA isolated from the leaves of seven hygromycin resistant plants. *Lanes*: *Pl* pCAMBIA1301 DNA showing hybridization signal at 11.8 kb, *C* Non-transformed control plant had no hybridization signal, *R1–R7* Seven putative transgenic plants that were rooted on hygromycin, out of them R4, R5, and R7 with hybridization signal are true transgenic plants.

8. Wrap the membrane with Kim wipe and expose the membrane to an imaging screen for 12 h and scan in Phosphorimager (*see* **Note 29**).
9. Lanes with radioactive signals indicates the presence of GUS gene in the corresponding plants **(Fig. 7)**.

4. Notes

1. Avoid contact of skin to the bleach. Commercial bleach can vary in active chlorine content. Make sure to check the product label and adjust dilution to obtain a 1.5% *(w/v)* final concentration of active chlorine.
2. Work as quick as possible to avoid desiccation of the plantlets.
3. Callus can be obtained using many other combinations of auxins and cytokinins.
4. We have successfully produced organogenic nodules from the callus that were induced in CIM, but there is no reason to believe that organogenic nodules can not be produced from callus obtained on other media.
5. If the culture flasks left unshaken for few minutes, organogenic nodules settles down and the medium can be simply removed without the need of steel mesh.
6. We use sterile 10.0 mL serological pipes (Sarstedt) in order to manipulate organogenic nodules along with the medium.
7. Because the organogenic nodules are delicate tissues, the period of osmotic treatment can be reduced and should not exceed 4 h. Longer osmotic treatments generally results in drastic reduction of plant regeneration.
8. To transfer 1.0 mL ONS onto filter paper discs, P-1000 micropipet with 1-mL tip with cut at the end can be used.
9. Since bacterial culture is initiated directly from the glycerol stocks, broth may grow slowly. By increasing the inoculum, optimal growth can be achieved overnight.
10. Alternatively, plasmid can be dissolved in sterile water. However TE preserves the plasmid for longer storage.
11. We generally sterilize the gold particles on the day before bombardment, although gold particles can be stored in glycerol for 1 wk.
12. DNA precipitation onto the macrocarrier is a critical step. After the addition of $CaCl_2$, spermidine should be added quickly to the mixture.
13. It is advisable to spread the microcarriers onto the macrocarriers immediately, as ethanol evaporates quickly. Ice incubation only prevents evaporation to a certain extent.
14. We prepare the whole setup a day before bombardment leaving the UV lights on overnight.
15. We keep rupture disks, macrocarriers, macrocarrier holders and stopping screens separately in sterile Petri dishes filled

with the sterilent either isopropanol or ethanol and dry out in the hood on a blotting paper before use.

16. Pipetting of the DNA-microcarrier mix onto the macrocarrier should be performed very rapidly in order to avoid agglomeration of the gold particles.
17. Although transformation of ONS cells can also be achieved with other flying distances, 9-cm flying distance show the best results in our experience. Do not forget to remove the Petri dish lid.
18. The vacuum pump should be powerful enough to reach the 28 mmHg value in 15 s. Regeneration is drastically affected if ONS are left under vacuum for longer period.
19. Post bombardment osmotic treatments exceeding 4 h will lead to the drastic reduction of explant survival.
20. The effect of medium containing hygromycin (SEL) on non-transformed tissues is quite obvious and leads to their death within 20 d.
21. Callus formation from the bombarded explants normally takes about 10 wk of culture on SEL.
22. This step can be avoided for cultures with elongated shoots.
23. Direct exposure of micropropagated *H. perforatum* plants to ambient conditions will lead to quick desiccation. Hence, maintaining the plants in water avoid desiccation and make them hard enough to withstand further acclimatization processes.
24. During this process, the delicate root system should not be damaged. We pass tap water with pressure through the medium which will allow the separation of plants from the medium without damaging the roots.
25. Growth chamber is not required if a greenhouse with appropriate lighting, temperature and humidity is available.
26. Use tissues from non transformed plants as negative control.
27. If not using radioactive labelled molecular weight marker, photographing the gel after electrophoresis will be useful to estimate the molecular weights of the hybridization signals.
28. Membranes can be stored at room temperature for several months before radioactive hybridization.
29. Exposure time should be adjusted based on the signal intensity of the hybridized membrane. As the positive control plasmid exhibits high signal, we cut the corresponding area and expose it separately.

Acknowledgments

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