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# Production of transgenic *Hypericum perforatum* plants via particle bombardment-mediated transformation of novel organogenic cell suspension cultures

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**Abstract**

We have developed particle bombardment-mediated transformation procedure for *Hypericum perforatum* L. (St. John's wort), an important medicinal species that remains highly recalcitrant towards *Agrobacterium*-mediated transformation. Among the major transformation techniques evaluated in the present study (*Agrobacterium tumefaciens*-, *A. rhizogenes*- and biolistics-mediated), particle-bombardment-mediated gene transfer was found to be the most successful one. GUS positive cells were obtained from organogenic nodules bombarded with the plasmid vector pCAMBIA1301 encoding an intron-containing β-glucuronidase (*gus*A) and hygromycin phosphotransferase (*hpt*) genes. After 3 months of continuous selection of bombarded nodules with 20 mg $l^{-1}$ hygromycin, transgenic hygromycin-resistant callus cultures and subsequently transgenic plants were produced. PCR analysis of DNA isolated from GUS positive plants showed the presence of both *gus*A and *hpt* genes. Southern blot analysis confirmed the transgene integration and revealed diverse copy numbers and insertion sites. The data presented here demonstrate for the first time *H. perforatum* can be efficiently transformed via particle bombardment of organogenic cell suspension. Our results open the possibility of using particle bombardment-mediated transformation to elucidate biosynthetic pathways and to improve secondary metabolite production in *H. perforatum*.


*Keywords:* *Hypericum perforatum*; Organogenic nodular structures; *Agrobacterium* transformation; GUS gene; Particle bombardment

## 1. Introduction

*Hypericum perforatum* L. (HP), commonly known as St. John's wort is an important medicinal plant used in the treatment of several pathologies since ancient times. The clinical efficacies of the HP extracts in the therapy of mild to moderate depressions have been confirmed in recent studies [1,2]. Many other important pharmaceutical properties of HP including antiviral [3], anticancer [4], neuroprotective [5] and antioxidant [6] activities have also been reported. Since treating humans and animals with HP extracts does not show any serious adverse side effects [7], use of this medicinal herb has increased dramatically during the past decade. Today, HP products are one of the top selling herbal medicines worldwide which are sold in the USA as a dietary supplement, while in Europe as anti-depressive agents [8]. These important pharmaceutical properties of the HP secondary metabolites have been the main thrust for the enormous research with cell cultures focused at present [9–13].

The use of plant cell and tissue cultures for the large-scale production of secondary metabolites has so far achieved only limited success due to the low and unreliable yields of the secondary products. Although significant improvements in product yields have been achieved through conventional biochemical approaches and the manipulation of the culture and process factors, the reproducibility of results is still a major concern [14]. Metabolic engineering is envisaged as an

*Abbreviations:* AS, acetosyringone; AVG, aminoethoxyvinylglycine; BA, 6-benzylaminopurine; BHT, butylated hydroxytoluene; FDA, fluoresein diacetate; GUS, ß-glucuronidase; HP, *Hypericum perforatum*; hpt, hygromycin phosphotransferase; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; MS, Murashige and Skoog; NAA, naphthaleneacetic acid; ONS, organogenic nodular structures; PGR, plant growth regulator

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effective and powerful tool for improving the biosynthesis of therapeutically useful compounds in medicinal plants [15,16]. As the pharmacological activities of HP extract are largely attributed to hypericin and hyperforin which are exclusively produced in this species, improving their production is an important target for genetic manipulation. This goal could not be achieved so far because of the poor knowledge about their biosynthesis and also due to the absence of transformation systems. Establishment of procedures for genetic transformation of HP would be useful for studying the biochemical and gene expression profiles of the biosynthetic pathways, and in metabolic engineering. Hence, there is an immediate need for establishing a transformation system for HP.

Among the several gene transfer techniques currently in practice, *Agrobacterium tumefaciens*-mediated transformation is the most efficient and commonly used technique in plant genetic engineering. On the other hand, hairy root cultures established by *Agrobacterium rhizogenes*-mediated transformation often sustain stable productivity in hormone-free culture conditions resulting in large amounts of secondary metabolites accumulation [17]. Particle-bombardment technique has been successfully used in the genetic transformation of a wide variety of plant species including many that are not amenable to *Agrobacterium*-mediated transformation [18] and become the second most widely used technique for plant transformation [19].

In the current investigation, we evaluated the efficiency of *A. tumefaciens*, *A. rhizogenes* and particle-bombardment techniques for transformation of HP. We report the successful genetic transformation of HP via particle-bombardment-mediated gene transfer for the first time.

## 2. Materials and methods

### 2.1. Sources of explants and plant regeneration

Various plant parts such as leaf blade, petiole, stem and root segments excised from the aseptic seedlings of HP var. Helos (purchased from Richters, Goodwood, Canada) were used as differentiated explants. The regeneration protocol adopted for these explants has been described elsewhere [20]. Briefly, explants obtained from 15-day-old aseptic seedlings were cultured on MS medium [21] containing 0.5 mg $l^{-1}$ benzylaminopurine (BA) and 1.0 mg $l^{-1}$ indole-3-acetic acid (IAA) and maintained at 16 h photoperiod.

As a source of novel organogenic nodule (ONS) explants, cell suspension culture was established from compact callus induced from HP (var. Helos) leaf explants as described earlier [22]. The established suspension cultures possessed morphologically distinct white and green cell types. The later cell type developed into ONS of about 1–3 mm in diameter (Fig. 1A) which resembled raspberry fruit (Fig. 1B). These ONS were filtered from the culture with a steel mesh screen (#40, Sigma, Barcelona) and cultured separately in Erlenmeyer flasks containing MS liquid medium supplemented with 0.475 mg $l^{-1}$ NAA on a rotary shaker at 80 rpm. For maintenance and multiplication, 10 ml of the suspension (medium + ONS) was transferred to Erlenmeyer flasks (250 ml) each containing 70 ml of medium and subcultured once in every 30 days. MS media supplemented with various concentrations of BA (0, 0.1, 0.5, 1.0 and 2.0 mg $l^{-1}$) and NAA (0.0 or 0.1 mg $l^{-1}$) were tested for plant regeneration from ONS. Culture plates inoculated with ONS were maintained in the dark until the onset of callus or shoot formation and thereafter transferred to photoperiod conditions.

Combination of 3% (w/v) sucrose and 0.8% (w/v) bacteriological grade agar (Sigma, Barcelona) was common to all solid media. The pH was adjusted to 5.8 (unless otherwise mentioned) before autoclaving at 1.0 kg $cm^{-2}$ for 15 min in both liquid and solid media. For callus/shoot induction, disposable Petri dishes containing 20–25 ml of solidified medium were inoculated with 5–10 explants, sealed with parafilm and cultured. All the cultures were incubated at 25 °C under photoperiodic (16 h) conditions under fluorescent bulbs (Fluora, Germany) at the light intensity of 25 μmol $s^{-1}$ $m^{-2}$ unless otherwise mentioned. Half-strength MS medium with 0.5 mg $l^{-1}$ indole-3-butyric acid (IBA) was used for rooting.

### 2.2. Optimization of hygromycin selection

To determine the appropriate concentration of hygromycin for the selection of transformants, explants were cultured on their respective regeneration medium, i.e. MS + 1.0 mg $l^{-1}$ BA + 0.1 mg $l^{-1}$ NAA for ONS (medium optimized in the

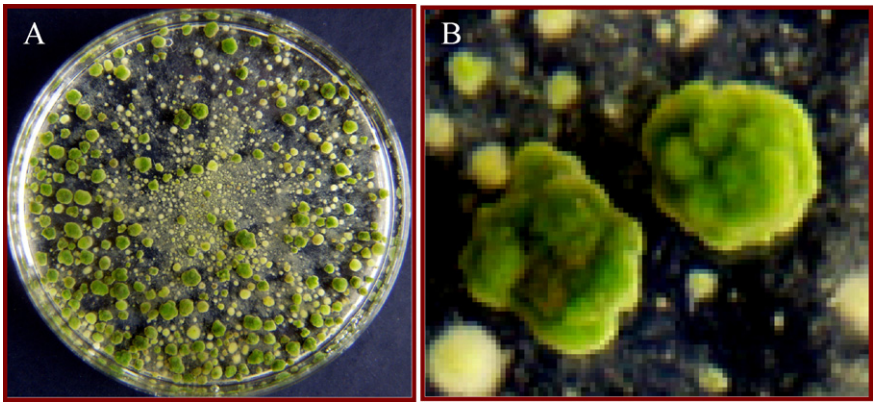


Fig. 1. (A) Organogenic cell suspension culture showing ONS explants. (B) Close-up view of ONS showing raspberry-like appearance.

Table 1
Regeneration of shoots from onganogenic nodules on MS medium supplemented with various concentrations of plant growth regulators after 30 days of culture initiation

| PGR (mg $l^{-1}$) | | Nodules regenerating (%) | Mean no. shoots ± S.D. per explant |
|---|---|---|---|
| BA | NAA | | |
| 0.0 | 0.0 | 0 | 0 |
| 0.1 | 0.0 | 10 | 4.5 ± 0.9a |
| 0.5 | 0.0 | 20 | 7.8 ± 1.5b |
| 1.0 | 0.0 | 40 | 8.4 ± 1.8b |
| 2.0 | 0.0 | 55 | 3.4 ± 1.2a |
| 0.0 | 0.1 | 0 | 0 |
| 0.1 | 0.1 | 60 | 11.3 ± 2.3b |
| 0.5 | 0.1 | 70 | 18.3 ± 1.9c |
| 1.0 | 0.1 | 90 | 25.6 ± 4.4d |
| 2.0 | 0.1 | 75 | 7.5 ± 1.8b |

Values followed by the same letters are not significantly different ($P > 0.05$, Kruskal–Wallis test).

present study, Table 1) and MS + 0.5 mg $l^{-1}$ BA + 1.0 mg $l^{-1}$ IAA for leaf blade, petiole, stem and root segments (medium optimized previously [20]) supplemented with different concentrations of hygromycin (0.0, 5.0, 15.0, 20.0, 30.0 and 50.0 mg $l^{-1}$). The ONS tissues were maintained in dark, whereas all other explants were maintained under photoperiod. Explants were maintained for 40 days with frequent subculture onto fresh medium once in every 10 days. The viability of explants was monitored by visual observation and fluorescein diacetate staining during each subculture.

### 2.3. Plasmid vector, Agrobacterium strains and culture

The plasmid pCAMBIA1301 (CAMBIA, Australia) containing the selectable *hpt* gene that encodes hygromycin phosphotransferase and the reporter *gus*A gene disrupted by catalase intron that favors the expression only in eukaryotic cells was used in all the transformation experiments. Both these genes are driven by CaMV 35S promoter, and are cloned in opposite orientation (Fig. 2). For *Agrobacterium*-mediated transformation, pCAMBIA1301 was transferred to the disarmed *A. tumefaciens* strains LBA4404 and EHA105 using $CaCl_2$ method [23]. *A. tumefaciens* strains were maintained in LB medium supplemented with 25 mg $l^{-1}$ rifampicin and 50 mg $l^{-1}$ kanamycin. Wild type *A. rhizogenes* strains A4 and LBA9402 were grown in MYA and YMB media, respectively.

Single *Agrobacterium* (*tumefaciens/rhizogenes*) colony was inoculated into 5.0 ml of appropriate liquid bacterial culture medium augmented with suitable antibiotics if required and incubated at 28 °C in a rotary shaker at 200 rpm for 12–16 h. Subsequently, 0.5 ml of grown bacterial broth was transferred to 250 ml Erlenmeyer flask containing 100 ml of bacterial culture medium and maintained under similar conditions. When the bacterial culture reached the optical density (OD) of 0.8–1.0 at 660 nm, bacteria were spun down using a tabletop centrifuge (Eppendorf, USA) at 4000 rpm and re-suspended in the *vir* gene induction medium [1× AB salts [24], 2 mM $NaPO_4$, 50 mM 2-morpholinoethanesulfonic acid (pH 5.6), 0.5% glucose and 100 μM acetosyringone (AS)].

### 2.4. Agrobacterium-mediated transformation

Leaf blade, petiole, stem and root segment explants were precultured in MS + 0.5 mg $l^{-1}$ BA + 1.0 mg $l^{-1}$ IAA medium and ONS were precultured in MS + 1.0 mg $l^{-1}$ BA + 0.1 mg $l^{-1}$ NAA medium for 24 h. Precultured explants were infected with the bacterial suspension for 5, 10, 20 and 30 min, blot-dried and transferred onto co-cultivation (CC) medium (regeneration medium + 200 μM AS, pH 5.2) for co-cultivation. To check the efficacy of antioxidants and ethylene inhibitor on T-DNA transfer, the co-cultivation medium was supplemented with 10.0 mg $l^{-1}$ butylated hydroxytoluene (BHT), 400 mg $l^{-1}$ cysteine, 100 mg $l^{-1}$ $AgNO_3$ and 5.0 mg $l^{-1}$ aminoethoxyvinylglycine (AVG) in different experiments. Explants co-cultivated with *Agrobacterium* (*tumefaciens/rhizogenes*) were thoroughly washed with sterilized distilled water to remove *Agrobacterium* contamination and transferred to regeneration medium under selection (with 20 mg $l^{-1}$ hygromycin and 250 mg $l^{-1}$ ticarcillin clavulanate) or without selection (only with ticarcillin for bacteria elimination).

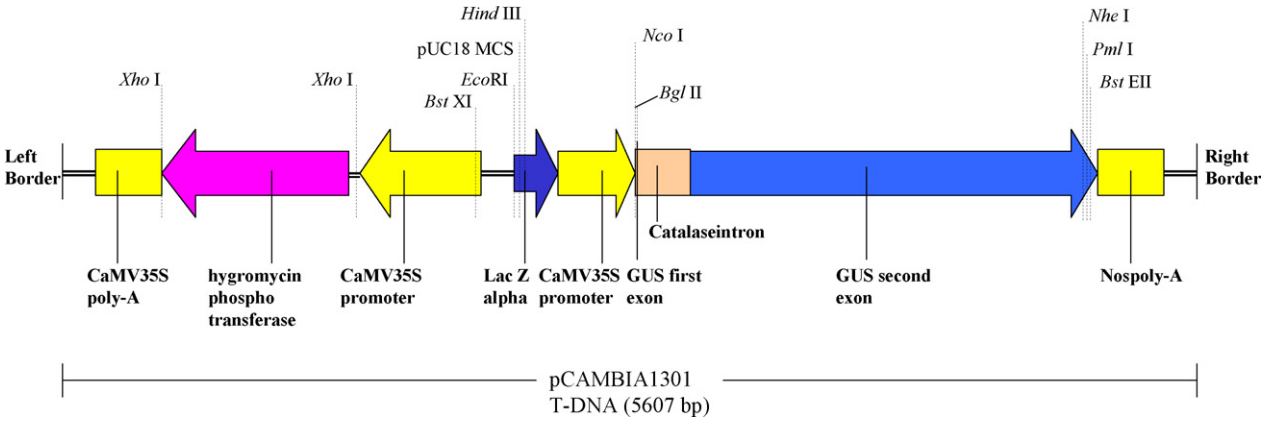


Fig. 2. T-DNA of pCAMBIA1301 showing restriction sites.

### 2.5. Plasmid isolation, precipitation of plasmid DNA onto gold particles and preparation of explants for bombardment

Plasmid DNA (pCAMBIA1301) was isolated from the *E. coli* strain (DH5α) using the Wizard® plus midipreps DNA purification system (Promega, USA) and precipitated onto 1.0-μm gold particles (Bio-Rad, USA) following standard procedures. Briefly, the following components were orderly added to an Eppendorf tube (1.5 ml) containing 87.5 μl of gold particles (7 mg in 50% glycerol) in agitation: 5 μl of plasmid DNA (1 μg/μl), 87.5 μl of 2.5 M $CaCl_2$ and 35 μl of 100 mM spermidine. The mixture was rested on ice for 10 min before centrifugation at 6000 rpm in a microfuge. The supernatant was discarded and the pellet was resuspended in 100 μl ethanol (100%). Just prior to loading the particle bombardment apparatus, the particles were vortexed for a few seconds to disperse any clumps. For each bombardment, 10 μl of DNA-gold suspension containing 700 μg gold particles associated with 0.5 μg plasmid DNA was spread and air-dried onto a macrocarrier.

Based on the results with *Agrobacterium*-mediated transformation, only ONS explants were selected for further experiments with particle bombardment. Approximately 4 h before bombardment, ONS were harvested from the suspension culture and placed in the liquid osmotic medium (MS + 1.0 mg $l^{-1}$ BA + 0.1 mg $l^{-1}$ NAA + 34 g $l^{-1}$ mannitol + 34 g $l^{-1}$ sorbitol). For each bombardment assay, 1.0 ml of ONS along with osmotic medium was poured at the center (3 cm diameter) of a sterile round Whatman filter paper disk until excess liquid was absorbed. Each disk was then carefully transferred to Petri dish (100 mm) containing 20 ml of solid osmotic medium before bombardment.

### 2.6. Particle bombardment

The bombardment chamber of a particle delivery system (PDS-1000/He, Bio-Rad) was evacuated at a pressure of 28 in. of mercury. Explants were bombarded with DNA-coated gold particles discharged with different rupture disk pressures (650, 900 and 1100 psi) from 9.0 and 13.0 cm flying distances (distance between stopping screen and target tissue) once. To check whether repeated bombardments can improve transformation efficiency, ONS were also bombarded twice (first and second hits from 9.0 and 13.0 cm, respectively) both with same rupture disk pressures (650, 900 or 1100 psi). Four hours after bombardment, the ONS were transferred onto regeneration medium. After 2 days incubation on regeneration medium, half of the bombarded explants were transferred to selection medium (MS + 1.0 mg $l^{-1}$ BA + 0.1 mg $l^{-1}$ NAA + 20.0 mg $l^{-1}$ hygromycin + 250 mg $l^{-1}$ ticarcillin clavulanate) and the other half were transferred to regeneration medium supplemented with 250 mg $l^{-1}$ ticarcillin clavulanate. The broad spectrum antibiotic, ticarcillin clavulanate was added in the media to prevent contamination.

### 2.7. Regeneration of transgenic plants

Calluses with shoot initials developed from the ONS explants on regeneration medium with or without selection were transferred to MS basal medium with hygromycin (20 mg $l^{-1}$) for shoot elongation. Elongated shoots (3–5 cm) were excised from the explant and transferred to Baby Food Jars containing 50 ml of half-strength MS medium supplemented with 0.5 mg $l^{-1}$ indole-3-butyric acid (IBA) and 20 mg $l^{-1}$ hygromycin for root induction. For hardening, culture bottles containing rooted plants were filled with sterile distilled water and incubated at room temperature (26 °C). After 2 days, the plants were removed from the medium, washed thoroughly in running tap water to remove the traces of rooting medium and planted in JIFFY-7 (Lisbon, Portugal) plant propagation systems and covered with polyethylene bags. The plants were acclimatized by reducing the humidity through making holes in the polyethylene bags and exposing to sunlight gradually for 1 week. Hardened plants were transferred to garden pots containing soil: compost (1:1), irrigated regularly with tap water and grown in a growth chamber (Fitotron, Sanyo) under photoperiod at 26 °C day–night temperature with 70% relative humidity.

### 2.8. Enzymatic histochemical GUS assay

GUS assay was performed periodically (after 2, 10 and 90 days) for explants from various experiments to monitor transformation. The percentage of initial transient expression was calculated as the number of explants showing blue spots divided by the total number of explants assayed and multiplied by hundred. The presence of the GUS gene in the putative transformants was detected histochemically following the procedure of Jefferson et al. [25]. Briefly, leaves and roots from plants obtained under early and late selection were incubated in a solution containing 100 mM $NaH_2PO_4$ buffer, pH 7.5, 50 mM each of $K_3FeCn_6$ and $K_4FeCn_6$, 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylammonium salt (Sigma) and 0.1% Triton-X 100 overnight at 37 °C and cleared in 70% ethanol.

### 2.9. PCR analysis of putative transgenic plants

Genomic DNA from the plants was isolated using DNeasy plant mini kit (QIAGEN, Germany). The *gus*A gene fragment was amplified using forward primer sequence 5′GATCGCGAAAACTGTGGAAT3′ and reverse primer sequence 5′TGAGCGTCGCAGAACATTAC3′. The forward and reverse primer sequences for the *hpt* gene amplification were 5′ATTTGTGTACGCCCGACAGT3′ and 5′GGATATGTCCTGCGGGTAAA3′, respectively. The reaction mixture contained 50 ng of genomic DNA, 2.0 μl of each primer (5 pmol), 0.5 μl of dNTP mix (2.5 mM each), 2.5 μl of PCR buffer, 0.25 μl of Taq DNA polymerase (5 U/μl) and the volume was adjusted to 25 μl with sterile distilled water. The PCR conditions included hot start at 94 °C for 4 m, followed by 30 cycles of denaturation (94 °C, 1 m), annealing (55 °C, 2 m) and extension (72 °C, 2 m), with a final extension of 10 m at 72 °C. PCR amplified products were resolved in 0.8% agarose gel with ethidium bromide.

### 2.10. Southern blot analysis

For Southern blot analysis, 20 μg aliquot of genomic DNA was digested with the restriction endonuclease *Eco*R I, electrophoresed on 1.0% agarose gel and transferred onto a Hybond nylon membrane (Amersham, UK). The pCAMBIA1301 DNA was used as the positive control. Prehybridization and hybridization were performed, respectively, for 3 and 16 h in church buffer (250 mM sodium phosphate buffer (pH 7.2), 1% BSA, 7% SDS and 1 mM EDTA) at 55 °C. PCR fragment of the *gus*A gene (1.3 kb) was labeled with α-[$^{32}$P] dCTP (Amersham, UK) using Prime-a-Gene® labeling kit (Promega, USA) and used as probe. Hybridized blots were washed twice with 2× SSC + 0.1% SDS each for 15 min and with 0.1× SSC + 0.1% SDS for 5 min at 55 °C. The blots were exposed to the imaging screen for 12 h and scanned in a Phosphorimager (Bio-Rad, USA).

### 2.11. Data analysis

For all the treatments, 3 independent experiments were done each with at least 10 samples. All the data were analyzed statistically. Regeneration from ONS under different combinations of plant growth regulators (PGRs) was analyzed by Kruskal–Wallis test followed by Dunn's multiple comparison test. The effect of rupture disk pressures and target distances on transformation was analyzed by one-way analysis of variance (ANOVA). The homogeneity of variance was tested by Cochran's test. Post hoc comparisons were made with the HSD Tukey test. All analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California, USA).

## 3. Results and discussion

### 3.1. Organogenic cell suspension cultures as source of explants

Several regeneration protocols have been reported for HP so far [26–29]. Recently, we have developed an efficient genotype-independent regeneration system from several explant tissues such as leaves, root segments and stem segments [20]. In spite of the robustness of this regeneration protocol, we were unable to achieve HP transgenic shoots from *Agrobacterium* transformations since the bacteria failed to infect any of the differentiated explants (leaf blade, petiole, stem and root segment). The recalcitrance of these explants to *Agrobacterium* infection might be due to the presence of hypericin and hyperforin, which are known antibacterial compounds [30]. Since, these compounds are only found in the differentiated tissues, in the present work, we have tested in comparison the efficiency of *Agrobacterium* and particle bombardment-mediated transformation in a novel explant, the green nodular structures produced from cell suspension cultures. Vardapetyan et al. [31] first reported the accumulation of globular structures in the later stages of HP cell suspension cultures which resembled raspberry fruit. However, the utility of these structures in regeneration and transformation of HP has only now been demonstrated.

### 3.2. BA concentration and light conditions affected plant regeneration from ONS

Plant regeneration from the ONS explants was affected by the BA concentration in a dose-dependent manner. There was a positive relationship between the percentage of ONS explants showing regeneration and BA concentration until 1.0 mg $l^{-1}$ (Table 1). Addition of NAA (0.1 mg $l^{-1}$) along with BA further increased the percentage of regeneration, with an optimum concentration of 1.0 mg $l^{-1}$ BA and 0.1 mg $l^{-1}$ NAA. Our previous study with differentiated explants also suggested that for callus induction and subsequent regeneration, HP needs a high cytokinin/auxin ratio [20]. In the absence of growth regulators or with NAA as sole PGR, no shoot induction was observed. Incubation in continuous dark until formation of calluses (1–2 weeks) is critical for plant regeneration from ONS, as the explants turned brown and showed delayed callusing when cultured under photoperiod at the beginning of culture. On the other hand, this initial dark treatment was not essential for regeneration from differentiated explants [20].

### 3.3. HP explants are highly sensitive to hygromycin

When the explants were cultured on optimal regeneration medium supplemented with different concentrations of hygromycin, all the explants died within 10 days on medium containing hygromycin concentrations higher than 15 mg $l^{-1}$. Only 5% of the explants formed callus on medium containing 15 mg $l^{-1}$ hygromycin. Hence, 20 mg $l^{-1}$ of hygromycin was considered optimum for the selection of transformants. Hygromycin selection was originally applied for the transformation of monocotyledonous species but it also works efficiently for recalcitrant dicotyledonous species [32–35].

### 3.4. Agrobacterium did not infect differentiated tissues

Susceptibility of the plant cell towards *Agrobacterium* infection is the foremost requirement for T-DNA transfer. In the present study, all the tested differentiated explants (leaf blade, petiole, stem and root segment) were found to be resistant to *Agrobacterium* and T-DNA transfer did not occur. None, out of hundred explants from each treatment assayed for GUS showed blue spots regardless of selective or non-selective conditions. Hence, we did not continue the experiments with these explants further. There are several reports of high necrosis and poor survival rate of target plant tissues during the process of *Agrobacterium*-mediated T-DNA transfer in other species [36–38] as a consequence of plant's hypersensitive reaction to *Agrobacterium* infection. In the case of HP, differentiated tissues such as leaves, stem, flowers and roots generally contain antimicrobial secondary metabolites such as hypericin and hyperforin, which can be induced in response to biotic elicitation [39] suggesting that the inability of *Agrobacterium*

to grow and infect these explants could be due to antimicrobial activity of these compounds.

### 3.5. *Agrobacterium* infected ONS in a low frequency in the presence of butylated hydroxy toluene (BHT)

All the ONS explants turned brown within one day of co-cultivation with *A. tumefaciens*/*rhizogenes*. Browning of explants co-cultivated with *A. tumefaciens* became more intense under selection pressure eventually, resulted in necrosis within 10 days and none of the explants showed transient *gus*A expression or callus formation. However, under non-selective conditions, all the ONS co-cultivated with *A. tumefaciens* and *A. rhizogenes* regained their normal growth within 5 days and produced calluses as the control. Staining of cells from these calluses using fluoresein diacetate 10 days after *Agrobacterium* infection confirmed 100% cell viability. In spite of the browning occurred after *Agrobacterium* co-cultivation, the cells managed to survive upon subculture in medium with no selection pressure. Moreover, genomic DNA isolated from the explants co-cultivated with *A. tumefaciens* did not show any fragmentation indicates that the incompatibility of *Agrobacterium*-mediated transformation in HP is not due to necrosis induced by programmed cell death as reported for other species [40].

Among the one hundred ONS explants assayed for GUS after co-cultivation with *A. tumefaciens* strain EHA105 on CC + BHT medium, two explants exhibited GUS foci when cultured on medium without selection. Similarly, one of the explants infected with the other *A. tumefaciens* strain LBA4404 also showed GUS foci suggesting that *A. tumefaciens* can infect HP under special conditions but in a very low frequency. These positive results could be due to the use of a strong anti-oxidant combined with the novel explant (ONS) which are devoid of organ-specific compounds such as hypericin. ONS explants co-cultivated in the presence of other antioxidants and ethylene inhibitors did not show any GUS foci indicate that BHT is more effective in scavenging harmful reactive molecular species. It seems that BHT can be used as a potential antioxidant in other recalcitrant plant species also. Even though the calluses obtained from ONS under non-selective conditions after co-cultivating with *Agrobacterium* regenerated shoots as the control, none of them were transgenic. Hence, they were not analyzed further.

A number of species previously considered recalcitrant are now efficiently transformed by supplementing antioxidants [38,41] and ethylene inhibitors [42–44] in the co-cultivation medium. This is mainly because of the fact that oxidative burst or ethylene production during plant–*Agrobacterium* interaction could be suppressed by these scavengers. However, in our case the tested antioxidants and ethylene inhibitor in the co-cultivation medium did not prevent browning indicating that the browning may be connected to the cellular defense responses. Recently, it was demonstrated that plants can modulate their gene expression in response to *Agrobacterium* infection and that *Agrobacterium* can actually trigger the plant defense machinery [45]. Changes in the phenolic profile and darkening of HP suspended cells after elicitation with fungal biomass was also reported [46]. Hence, the browning response of HP when infected with *Agrobacterium* could be somewhat connected to the modulation of the phenylpropanoid pathway.

### 3.6. Rupture disk pressure and the number of bombardments affected the recovery of transgenic plants

In the present study, the initial transient expression level of ONS explants bombarded once (either 9.0 or 13.0 cm target distance) and twice (from both 9.0 and 13.0 cm target distances) remained similar. However, the callus induction frequencies were significantly reduced ($P < 0.05$) in ONS bombarded twice under selective and non-selective conditions (Fig. 3A and B). This might be attributed to the extent of mechanical injury created by gold particles and cell death when the same tissues were bombarded twice. When bombarded once, callus induction was higher in explants hit by gold particles released from 1100 psi rupture disks than 650 and 900 psi rupture disks under selection in both 9.0 and 13.0 cm flying distances (Fig. 3B, $P < 0.05$). This variation in callus induction frequencies between rupture disk pressures may be attributed to the dissimilar penetration rate of the gold particles and eventually to the DNA delivery, as the cells received the DNA only can survive under selection. Effective penetration of the target plant tissue by the microprojectiles carrying the DNA is essential for successful gene delivery. The flying distance and rupture disc pressure are obviously affecting the speed of the microprojectiles when reaching explant surface [47]. Rupture

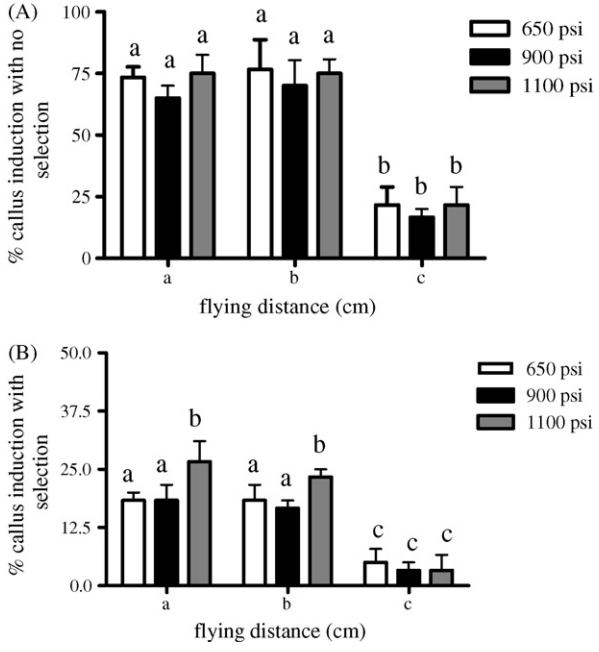


Fig. 3. Graphical presentation of the effect of rupture disk pressures (650, 900 and 1100 psi) and number of shots (a, single shot from 9.0 cm flying distance, b, single shot from 13.0 cm flying distance and c-two shots; one from 9.0 cm and the other from 13.0 cm flying distances) on DNA delivery. (A) Callus induction frequency of ONS 45 days after bombardment under non-selective condition. (B) Callus induction frequency of ONS 80 days after bombardment under selection. Error bars with different letters are statistically different ($P < 0.05$).

Table 2
Effect of the number of bombardments, rupture disk pressures and flying distances on HP transformation using ONS explants under early (ES) and late (LS) selection

| No. shots | Target distance (cm) | Rupture disk pressure (psi) | Number of explants | | PCR positive plants | | Transformation efficiency (%) | |
|---|---|---|---|---|---|---|---|---|
| | | | ES | LS | ES | LS | ES | LS |
| 1 | 9 | 650 | 32 | 33 | 0 | 0 | 0 | 0 |
| 1 | 9 | 900 | 32 | 36 | 1 | 0 | 3.1 | 0 |
| 1 | 9 | 1100 | 31 | 30 | 4 | 1 | 12.9 | 3.3 |
| 1 | 13 | 650 | 30 | 31 | 0 | 0 | 0 | 0 |
| 1 | 13 | 900 | 30 | 33 | 1 | 0 | 3.3 | 0 |
| 1 | 13 | 1100 | 30 | 32 | 2 | 0 | 6.7 | 0 |
| 2 | 9, 13 | 650 | 32 | 30 | 0 | 0 | 0 | 0 |
| 2 | 9, 13 | 900 | 32 | 31 | 0 | 0 | 0 | 0 |
| 2 | 9, 13 | 1100 | 30 | 38 | 0 | 0 | 0 | 0 |

disk pressures and flying distances did not have any effect on plant regeneration under both early as well as late selection. However, when verified by PCR, the combination of 9.0 cm flying distance with 1100 psi rupture disk pressure resulted in more transgenic plants than other treatments under both early and late selection (Table 2). This result indicates that the higher acceleration we tested to drive the microprojectiles was the most beneficial factor for HP transformation.

### 3.7. Timing of hygromycin selection critically affected transformation efficiency

Timing of hygromycin selection of the bombarded explants critically affected transformation efficiency (Table 2). When the explants were transferred to selection medium 2 days after bombardment, only after 10 weeks about 25% of explants produced callus from each plate (Fig. 4A). These calluses regenerated shoot buds after 6–9 months of bombardment, but in a very low frequency (3.0 shoots/callus). Eight shoots rooted on half-strength MS medium supplemented with 0.5 mg $l^{-1}$ IBA and 20 mg $l^{-1}$ hygromycin (Fig. 4F).

Under non-selective (late selective) condition, ONS proliferated during the first 2 weeks of culture, and callus began to appear 4 weeks after bombardment similar to the control. Two weeks later, about 90% of these explants had formed yellowish calluses with a characteristic red pigmentation (Fig. 4B), 55% of which regenerated shoots (Fig. 4C). Subsequently after 2–3 months, when transferred to selection medium, these cultures produced several albinos along with green shoots (Fig. 4D). In rice, late selection resulted in the formation of chimeric callus lines [48] while in colonial bent grass it produced albino plantlets [49]. Repeated selection of cultures with shoots finally resulted in the regeneration of uniform green shoots (Fig. 4E). Five green shoots produced roots on rooting medium with 20 mg $l^{-1}$ hygromycin.

PCR analysis of the genomic DNA isolated from all the eight rooted plants obtained from early selection had the expected fragments of *gus*A (1.3 kb) and *hpt* (0.8 kb) genes amplified (Table 2). Whereas, among the five rooted plants obtained from late selection, one had the expected fragments (Table 2). As expected, the transgenes were not amplified in the control plant. These results indicate that the early selection is efficient in producing transgenic plants without escape vice versa in late selection.

All the rooted plants were subjected to acclimatization process. However, a total of seven plants (R4 and R5 from early selection and R1, R2, R3, R6 and R7 from late selection) could be successfully hardened (Fig. 4G) and established in garden pots (Fig. 4H) as described earlier [20] for further analyses.

### 3.8. GUS histochemical evidence for transformation

Transient *gus*A gene expression 48 h after bombardment was used as an initial indicator of the efficiency of gene transfer (Fig. 5A). DNA delivery frequencies ranged from 45 to 75% of GUS-positive explants per bombarded plate. This high variability in transient gene expression between plates is probably due to the variations in the degree of the gold particles coating with the DNA as reported earlier [50], or to the aleatory distribution and penetration of gold particles, or even to variations in physiological status of the ONS. In all the GUS-positive explants, the foci were observed in the clustered multi-cell aggregates rather than single cells (Fig. 5A, inset). Individual GUS focus could be seen even after 10 days of bombardment (Fig. 5B) eventually indicating stable expression.

When the explants with slow-growing calluses obtained from early selection were subjected to GUS assay, blue staining was confined only to the newly formed calluses (Fig. 5C) indicating that growth of non-transformed tissues was completely prevented by this early selection. When the explants with callus and shoots, developed under non-selective conditions, were subjected to histochemical GUS test, blue staining was found in the calluses and in small parts of the shoots (Fig. 5D) indicating chimeric *gus*A gene expression.

All the plants established in the pots after early selection (R4 and R5) and one out of five from late selection (R7) stably expressed the *gus*A gene in roots (Fig. 5E) and leaves (Fig. 5F). Since genes interrupted with an intron will only express after intron splicing, and the non-transformed HP explants tested for GUS expression were negative (Fig. 5F right), the integration of the transgene in these three plants could be confirmed.

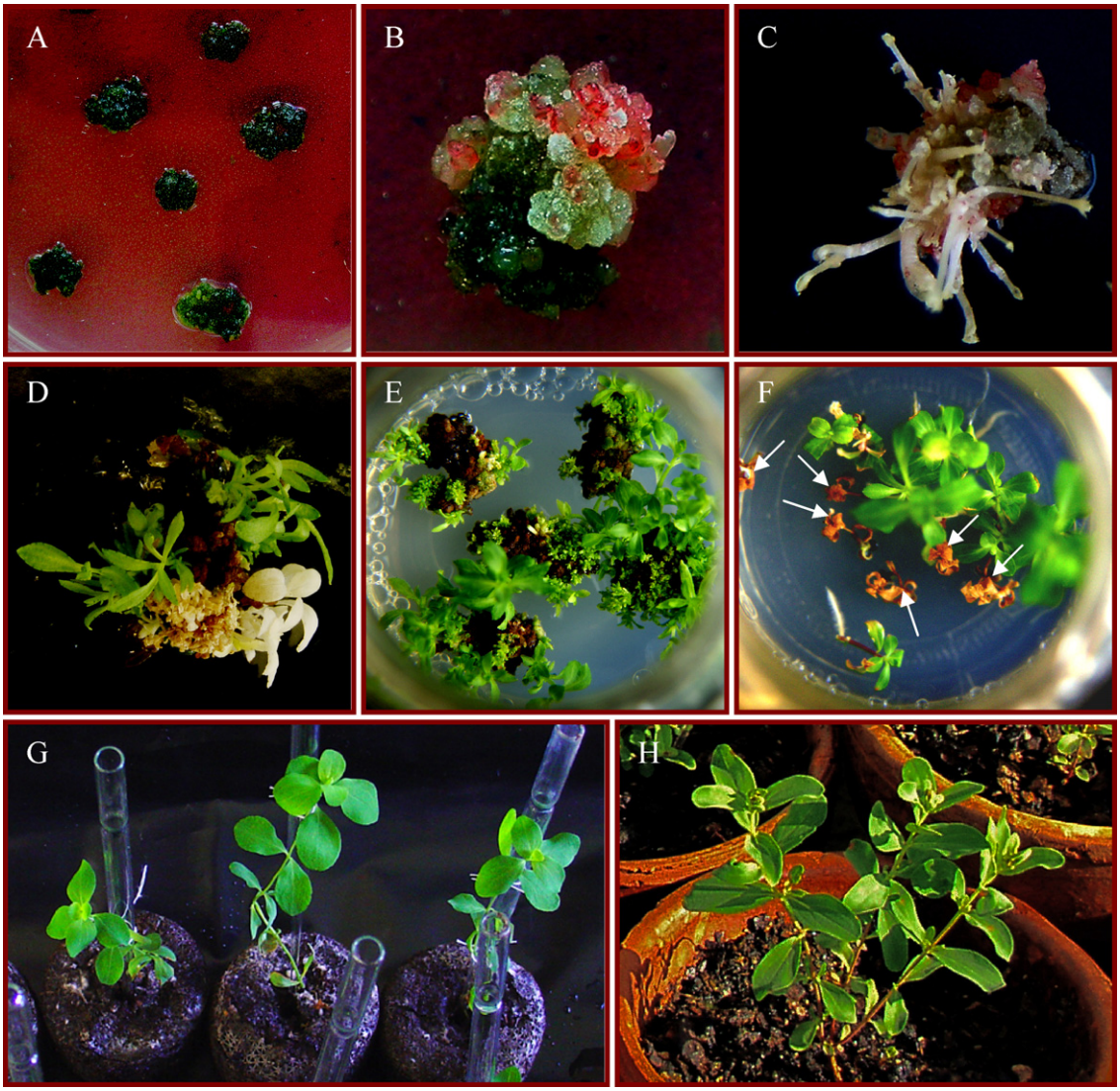


Fig. 4. Particle bombardment-mediated transformation of HP with pCAMBIA1301. (A) Callus development from bombarded explants after 10 weeks on selection medium. (B) Quick proliferation of yellowish calluses possessing characteristic red pigmentation from bombarded explants under non-selective conditions after 6 weeks of incubation under dark. (C) Shoots regenerating from explants under non-selective conditions after 8 weeks of dark incubation. (D) Cultures with albino and green shoots upon late selection (3 months) of cultures initially maintained under nonselective condition. (E) Cultures with uniform green plants after 3 subsequent selections. (F) Rooting of regenerated shoots under selection showing death of shoots (arrows) susceptible to hygromycin and root initiation from resistant ones after 12 days. (G) Hardening of putatively transformed HP plants. (H) A transgenic plant established in pot. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article).

### *3.9. Molecular evidence for transgene integration in the transgenic plants*

Southern blot analysis of the seven potted plants (R1–R7) is shown in Fig. 6. Among these, the three plants which were positive for GUS assay and PCR (R4, R5 and R7) had hybridization signals in the Southern blot which confirmed the presence of the *gus* gene in these transgenic HP plants. No hybridization signal was detected in genomic DNA from nontransformed control plant.

Southern blot analysis also revealed that the *gus*A gene had integrated into the HP genome of different individuals with diverse copy numbers and insertion sites. The observed band pattern indicates that the three transgenic plants were derived from three independent transformation events. The difference in the banding pattern between R4 and R5 is a point for different transformation events, although both shoots derived from the same callus. Each sample showed a different band pattern depending on where the T-DNA integrated in the plant genome, since only one *Eco* RI site was present in the T-DNA (Fig. 2). The Southern pattern showed eight or more fragments, thus representing hybrid molecules containing DNA from both the vector and the plant genome, and demonstrating transgene integration in different loci (eight to ten sites). The strong bands in the lanes of R4 and R7 probably indicate multiple copies of the transgene integrated as concatemers.

Biolistics-mediated transformation generally results in high copy number transgene integration [51] which may lead to

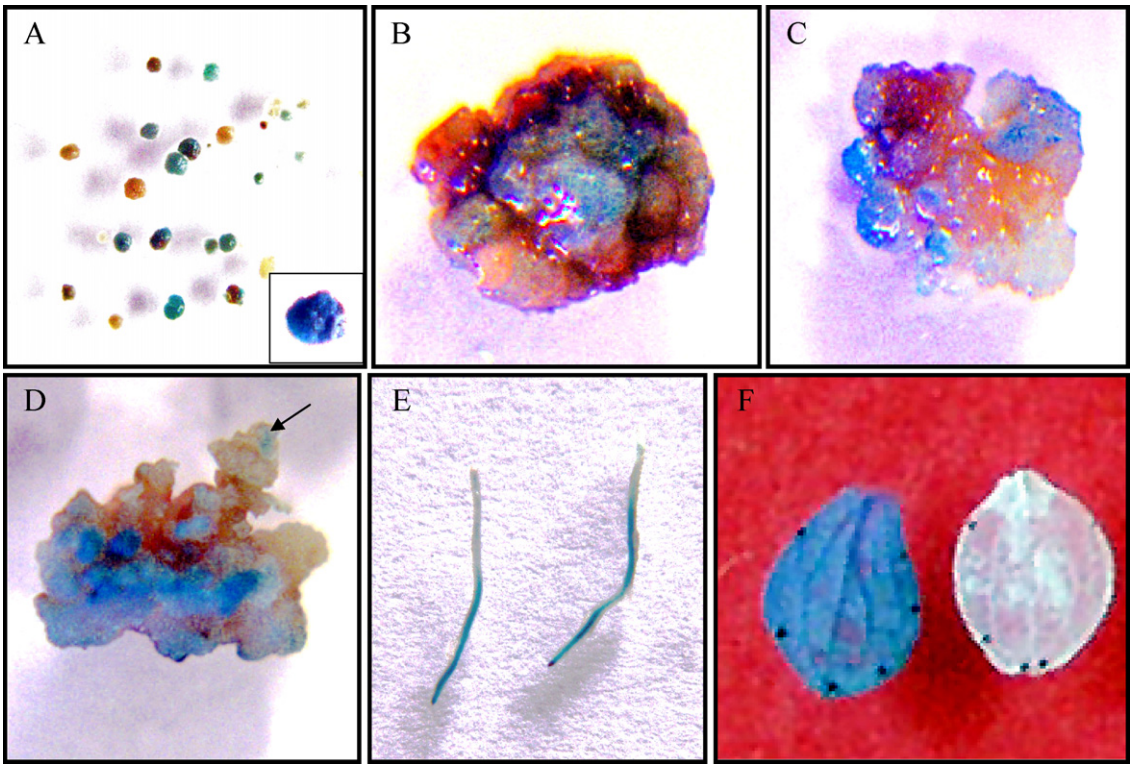


Fig. 5. Expression of *gus*A gene during the development of transgenic HP plants from the ONS. (A) A plate of ONS explants bombarded with pCAMBIA1301 DNA using 1100 psi rupture disk with 9.0 cm flying distance showing many explants with transient GUS expression and others with no expression (inset, close up view of an ONS showing clustered and indistinguishable GUS foci). (B) A GUS positive explant showing several GUS foci 10 days after bombardment indicating stable transgene expression, close up view. (C) An ONS explant showing GUS activity confined to the newly formed calluses under early selection after bombardment. (D) An explant subjected to hygromycin selection only after 3 months of bombardment showing GUS activity in the callus as well as in a portion of a shoot (arrow) indicating chimeric gene expression. (E) Root segments of a transgenic HP plant showing GUS activity. (F) Leaf of a transgenic HP plant showing GUS activity and control leaf in the right; note the characteristic feature of HP leaves, the presence of black spots indicating hypericin glands. (For interpretation of the references to *gus*A gene expression in this figure legend, the reader is referred to the web version of the article).

transgene silencing. From the Southern analysis of the transgenic HP plants, we found no correlation between transgene copy number and expression, since the transgenic HP plants stably expressing GUS are multicopy for GUS gene.

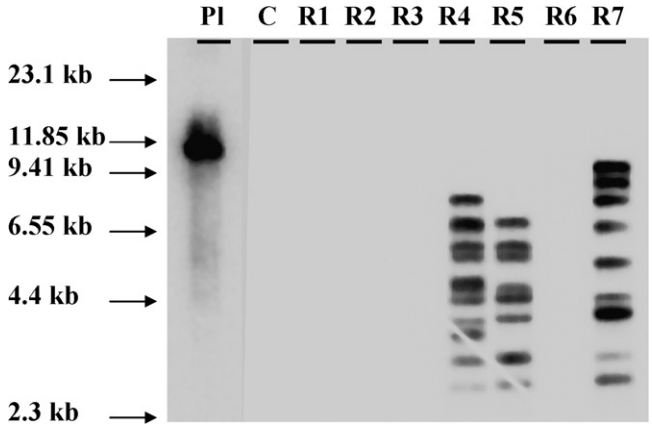


Fig. 6. Southern blot analysis of DNA isolated from leaves of non-transformed control plant and seven hygromycin resistant plants established in pots. (Pl) pCAMBIA1301 DNA showing hybridization signal at 11.8 kb. (C) Non-transformed control plant had no hybridization signal. (R1, R2, R3 and R6) Plants had no hybridization signal indicating late selection resulted in high frequency of escapes upon rooting on hygromycin. (R4 and R5) Plants with hybridization signal indicating early selection is efficient in eliminating escapes. (R7) Transgenic plant obtained from late selection.

Craig et al. [52] also demonstrated the lack of correlation between gene copy and/or insertion site number with gene expression levels in biolistically transformed potato lines. Similarly, transgenic rice lines that received multicopies of *GUS* and/or *bar* could express the gene(s) stably at high levels up to the R3 generation [53].

In conclusion, as the host range of *Agrobacterium* pose obstacles for the transformation of HP, particle bombardment can be successfully combined with the regeneration of shoots from ONS via organogenesis in producing transgenic HP plants. This lays the foundation for metabolic engineering of HP for the production of important secondary metabolites such as hypericin and hyperforin. The use of ONS as target tissue is a key factor for the recovery of transgenic HP plants in our study.

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