Examen Master Biologie, Agrosciences 07 Mai 2020

4TBA804U Biologie et Biotechnologies des Plantes (1h30, documents non autorisés)

Withania somnifera (**Ashwagandha)** est une espèce de la famille des ***Solanacées***. Elle fait partie de la pharmacopée traditionnelle d'Inde où elle est cultivée sur plusieurs milliers d'hectares. Les feuilles contiennent de nombreuses **withanolides**, des métabolites secondaires de type stéroïdes, possédant diverses propriétés pharmacologiques et constituant de potentiels candidats pour de nouvelles molécules à effet thérapeutique.
Depuis 2015, le CNRS a déposé cinq brevets concernant *Withania somnifera* afin de traiter ou prévenir les maladies neurodégénératives.

1) A la lecture de l'article suivant, quelles sont les étapes identiques au protocole de transformation stable du tabac que vous avez réalisé en travaux pratiques? (**55/200**).
2) Quelles sont les différences majeures des protocoles de transformations de Withania et de tabac et/ou les étapes du protocole en plus ou en moins pour transformer Withania? Proposez des hypothèses pour expliquer pourquoi il y a ces différences de protocoles (**55/200**).
3) La figure 1 décrit l'organisation génique du T-DNA portée par le plasmide binaire. Vous remarquerez que la construction porte un gène gusA avec un intron. Plus loin dans les résultats, les auteurs indiquent : « since the intron containing gusA gene can make active enzyme only in the transformed leaf tissues, and not *A. tumefaciens* ». Proposez un mécanisme qui permet d'expliquer ce résultat et pourquoi cette situation est importante pour l'interprétation des résultats par les auteurs **(30/200)**.
4) Mettez-vous dans la peau d'un reviewer d'article scientifique pour Cell Report pendant quelques minutes: quelles sont les 2 remarques (2 minimums) que vous feriez aux auteurs dans votre rapport concernant les « Supplementary material Fig. S1 et S2» pour améliorer lesdites Figures S1 et S2? (**30/200**).
5) Supposons que les auteurs concluent qu'ils ont réussi à transformer Withania (ce qui semble être le cas), proposez une stratégie pour exploiter ces résultats pour une application biotechnologique ou autre? **(30/200)**.

Plant Cell Rep (2010) 29:133–141
DOI 10.1007/s00299-009-0805-0

ORIGINAL PAPER

Agrobacterium tumefaciens-mediated transformation of *Withania somnifera* (L.) Dunal: an important medicinal plant

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Materials and methods

Plant material and explant preparation

Seeds of six different accessions (RSS-1, RSS-4, RSS-8, RSS-16, RSS-30 and WSB) representing different chemotypes of *Withania somnifera* (L.) Dunal, were procured from CIMAP, Lucknow, India, and germinated in vitro following the procedure reported by Kulkarni et al. (1996). Seeds were given a cut with blade and inoculated on Murashige and Skoog's (MS 1962) medium for in vitro germination. The shoots were obtained on MS medium supplemented with 2.2 µM BA and 1.1 µM IAA. A parallel experiment was set up for seed germination under greenhouse conditions where seeds were sown in sterilized potted soil.

Leaves from GH-grown seedlings and in vitro-shoots were used as explants for cocultivation. The GH-grown leaves measuring approximately 2–3 cm in length and 1.5–2.5 cm in width were excised from first to fifth nodes from the shoot tip. The leaves were washed with tap water, surface-sterilized with 0.1% $HgCl_2$ for 3–5 min, followed by 3–4 washes with sterile distilled water. Leaves from in vitro-shoots were taken as such for cocultivation. From both types of leaves, midvein and margins of leaf lamina were removed to expose cut surfaces for infection. To determine the optimum age of GH-grown seedlings for transformation, 30 to 90-day-old seedlings were used for taking explants for cocultivation.

Bacterial strains and plasmid vectors

Agrobacterium tumefaciens strains LBA4404 and EHA101, containing the binary vector pIG121Hm (Fig. 1) were used for transformation. The T-DNA of pIG121Hm contains neomycin phosphotransferase (*nptII*) and hygromycin phosphotransferase gene (*hptII*) as selection markers and *gusA* gene containing intron as the reporter gene. The *nptII* gene is under the control of P*nos* (nopaline synthase promoter) and the *hptII* and *gusA* genes are under the control of cauliflower mosaic virus (CaMV) 35S promoters (Ohta et al. 1990). To optimize transformation conditions, LBA4404 with plasmid pBI121 having *gusA* reporter and *nptII* selection marker genes was also used.

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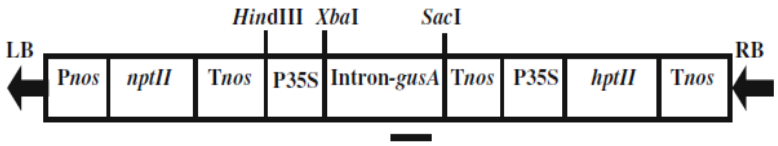

Fig. 1 T-DNA construct of binary vector pIG121Hm showing restriction sites and *gusA* with intron used for transformation of *W. somnifera*. *RB* right border, *Pnos* nopaline synthase promoter, *nptII* neomycin phosphotransferase gene, T*nos* nopaline synthase terminator, *P35SCaMV* Cauliflower Mosaic Virus 35S promoter, *gusA* intron containing *β*-glucuronidase gene, *hptII* hygromycin phosphotransferase gene, and *LB* left border of T-DNA. *Line* shown below the map represents region of the *gus* gene used for Southern blot analysis in this study

Transformation

Agrobacterium tumefaciens (LBA4404) was grown at 28°C in YEB minimal medium (Lichtenstein and Draper 1986) in the presence of 50 mg l^{-1} kanamycin, 250 mg l^{-1} streptomycin and 100 mg l^{-1} rifampicin. In case of EHA101, streptomycin was replaced with hygromycin. The primary and secondary cultures were grown in YEP medium at 28°C for 20–22 h at 200 rpm in incubator shaker with the same antibiotic concentrations as LBA4404. *A. tumefaciens* cells were harvested in log phase at $OD_{600} = 0.6$ by centrifugation and resuspended in liquid MS media (MSL) having 100 µM acetosyringone. The cells were kept in shaker incubator at 28 °C, at 150 rpm for about 30 min. Leaf sections were immersed in *A. tumefaciens* suspension for 10–30 min with gentle shaking and blotted dry on sterile filter paper. The infected leaves were placed on cocultivation medium and kept in the dark for different time periods ranging from 3 to 6 days. The cocultivated leaf sections were transferred to SIM for shoot bud induction containing 400 mg l^{-1} augmentin. Results of transient Gus expression were compared between directly infected and ultrasonicated (Bransonic® Ultrasonic Cleaner, USA, Model No. 3210E-MTH) leaf explants at 47 KHz ± 6% for 10 s.

Media composition and culture conditions

The CCM, containing the MS basal medium supplemented with 8.9 µM BA and 8.0 µM IAA was used. After cocultivation, 400 mg l^{-1} augmentin was used in CCM and the medium was named as shoot induction medium (SIM). The concentration of augmentin was gradually reduced to 200 mg l^{-1} in subsequent subcultures. In SIM, MIC for kanamycin (25, 50, 75 or 100 mg l^{-1}) was determined for the selection of transformed shoots in the presence of augmentin. After 2 cycles of 20 days each, in SIM, the explants were inoculated in the selection media having 50 mg l^{-1} kanamycin. During III and IV cycle, shoot buds started differentiating from the leaf segments. In V cycle, the concentration of growth regulators was lowered to BA (1.1 µM) and IAA (0.6 µM) for elongation of the regenerated shoot buds. This medium was named as Shoot Elongation Medium (SEM). Roots were induced in MS medium supplemented with 1.4 µM NAA, 50 mg l^{-1} kanamycin and 200 mg l^{-1} augmentin. After root induction, the plantlets were sub-cultured in the hormone-free and selection-free medium for further growth. The rooted shoots were acclimatized first in Hoagland's medium for 1 week and then transferred to sterilized potted soil. During initial 10–15 days of hardening, high humidity was maintained by covering the plants with acclimatization hood of plexiglass (Basco Pvt. Ltd., India) and irrigated with sterile water. For the next 15 days, acclimatization hoods were raised to decrease the humidity gradually. After 4 weeks, the plants were kept in green house at 25 ± 2°C. Initially, the plantlets were covered with polythene for 1 week and slowly the humidity was decreased by removing the polythene under greenhouse conditions.

The pH of all the tissue culture media was adjusted to 5.8 prior to the addition of agar and autoclaving. All the media were solidified with 0.8% agar. Cultures were incubated at 25 ± 2°C under 16/8 h light/dark period. Light at 60 µmol photon m^{-2} s^{-1} was supplied by fluorescent tube lights fitted on culture racks. All biochemicals and media constituents of molecular biology grade were procured from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

Gus histochemical assay

Transient Gus expression was seen in the leaf explants just after cocultivation, while stable Gus expression was seen in the kanamycin-resistant regenerated transgenic plants. Gus was assayed using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc, Biosynth) supplemented with 20% methanol, as described by Kosugi et al. (1990). This assay gives a blue colouration in the presence of the *β*-glucuronidase enzyme. Explants were tested by incubating overnight with the substrate (1% w/v in phosphate buffer, pH 7.4, supplemented with 0.01% Triton X-100) at 37°C. Stained tissues were rinsed extensively in 70% ethanol to remove residual chlorophyll.

Molecular analysis

PCR analysis of transformants

Plant genomic DNA was isolated using the DNeasy Plant Minikit, QIAGEN. The transformants were examined by PCR amplification of *nptII* and *gusA* genes. The oligonucleotide primers for *nptII* gene were 'forward': 5′CTG AAT GAA CTG CAG GAC GAG G 3′ and reverse: 5′ GCC AAC GCT ATG TCC TGA TAG C 3′ and those for *gusA* gene were 'forward': 5′ GAT CTG AGG GTA AAT TTC TAG TTT TTC TC 3′ and 'reverse': 5′TGG ATT CCG GCA TAG TTA AAG AAA T 3′. Amplification was carried out at 94°C for 5 min, 94°C for 1 min, 58°C (for *nptII*) and 60°C (for *gusA* containing intron) for 1 min as annealing, 72°C for 1 min as elongation, and 72°C for 5 min final extension. Amplicons were electrophoresed on 1.0 % (w/v) agarose gels.

Assay for Gus activity

The standard protocol of Jefferson et al. (1987) was followed for assaying Gus enzyme activity. Fresh leaf tissue (100 mg) was ground with 1 ml extraction buffer (50 mM Na_2HPO_4 pH 7.0, 10 mM DTT, 0.1% sodium lauryl sarcosine (w/v), Triton X-100, 10 mM Na_2EDTA) and mixed well for making homogenous mixture. The mixture was centrifuged at 13,000 rpm, at 4°C, for 5–10 min. Supernatant (90 µl) was added with 10 µl of 10 mM MUG (4 methyl-umbelliferyl-β-D-glucuronide). The mixture was then incubated at 50–55°C for 25 min and added with 25 µl methanol in the dark. The samples were incubated at 37°C for 2 h and the reaction was stopped with a tenfold volume of 0.2 M Na_2CO_3. The protein concentration was determined as in Bradford (1976), using bovine serum albumin as the standard.

RT-PCR analysis of transgenic plants

Total RNA was isolated using RNeasy® Plant Mini Kit, QIAGEN. Each RNA sample was treated with DNase (Fermentas) to eliminate DNA contamination. Five µg RNA was used as template for reverse transcription. The cDNA was synthesized using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. RNA samples without reverse transcription (RT minus) were included as control, to ensure the absence of contaminating DNA. RT-PCR reactions were performed similar to genomic DNA PCR reactions as described above, using cDNA template in each reaction. The PCR product was subjected to electrophoresis in 1% agarose gel. The actin gene was used as an internal control to check the expression of reporter genes.

Segregation analysis in T_1 progenies

T_1 plants were raised from selfed seeds of T_0 progeny germinated under GH-conditions. After 4–8 weeks, leaves of T_1 plants were used for Gus histochemical assay. The segregation analysis was done by counting the number of plants which were positive in PCR analysis over total number of plants. The inheritance of the *gusA* gene in plant progenies was confirmed by PCR.

Southern blot analysis

One representative from each of the transformed line, which gave positive results with PCR and Gus assay in T_1 generation, was analysed by Southern hybridization. The genomic DNA (20 µg) was digested with *EcoR1* and subjected to 0.7% agarose gel electrophoresis. The agarose gel was depurinated in 0.25 N HCl for 20 min till the colour of the dye turned yellow. The gel was then placed in denaturation buffer (15 min) until the colour of the dye turned blue again. The digested DNA was transferred to Hybond N^+ membrane (Sigma Chemical Company, St. Louis, MO) in denaturation buffer using the vacuum blot apparatus. The blot was probed using radiolabelled probe prepared by PCR of a part of the *gusA* gene.

Statistical analysis

The experiments were conducted in a completely randomized design. The data involving ten replicates of each treatment were used for statistical analysis. Standard errors of the means of ten replicates have been presented, while the experiment was repeated thrice.

Results

Effect of accession, explant source, position of leaf and seedling age on transformation

Transient Gus assay carried out with leaf explants after cocultivation showed distinct blue spots. These were used as a parameter for optimizing the conditions of transformation since the intron containing *gusA* gene can make active enzyme only in the transformed leaf tissues, and not *A. tumefaciens*. Different accessions of *W. somnifera* were assessed on the basis of their regeneration ability and transient transformation efficiency (TTE) calculated through histochemical Gus assay (Table 1). RSS-30 had the highest regeneration potential as well as Gus expression. Hence, detailed experiments were performed only with RSS-30. *W. Somnifera* seeds took about two-and-a-half months to germinate under in vitro conditions. In the soil, germination took only 15 days under the GH-conditions.

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Table 1 Shoot bud regeneration and transient transformation efficiency in leaf segments of different accessions of *W. somnifera*

Accession	Explants (%) regenerating shoots (Mean ± SE)[a]	Explants (%) expressing Gus[b]
RSS-1	77.7 ± 1.45	66.7
RSS-4	63.3 ± 0.88	–
RSS-8	52.3 ± 1.45	26.0
RSS-16	64.3 ± 2.34	–
RSS-30	95.0 ± 2.89	77.3
WSB	22.7 ± 1.45	45.0

[a] Average of 10 replicates 6 weeks after culture initiation

[b] Gus expression 5 days after cocultivation

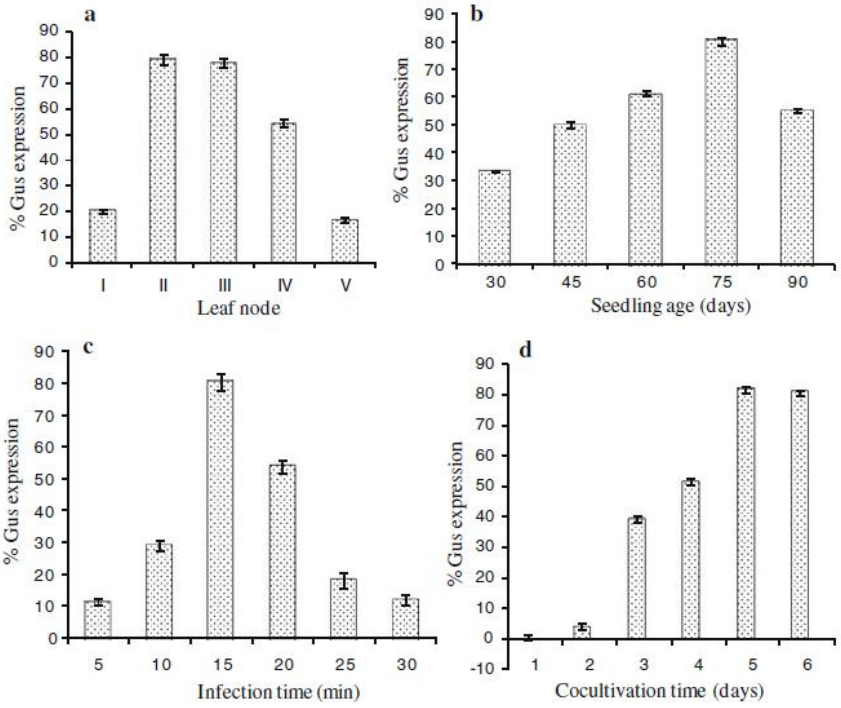


Fig. 2 Histochemical Gus expression in respect to (**a**) position of the leaf; (**b**) seedling age (days); (**c**) infection time (min); and (**d**) cocultivation time (days)

Fig. 3 Different stages of transformation in *W. Somnifera*. Leaf explants (**a**) after 40 days of cocultivation, ready to be used for selection; (**b**) during selection cycles of kanamycin; (**c**, **d**) different stages of shoot development after selection; (**e**) proliferation of kanamycin resistant shoots; (**f**) rooted plantlet;

Supplementary material Fig. S1

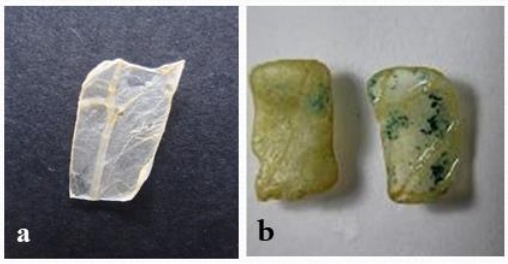
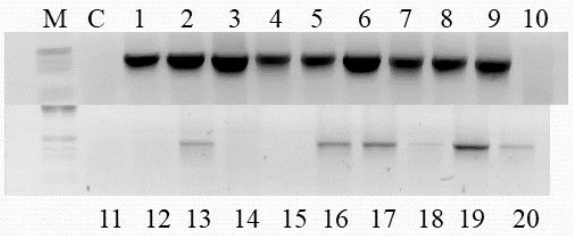
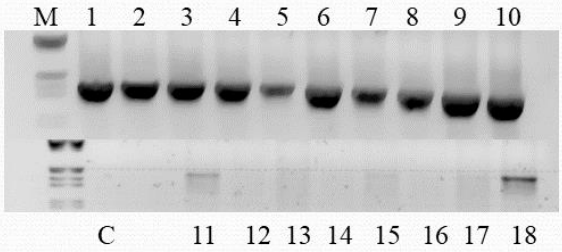


Fig. 1 Histochemical Gus expression in (a) control and (b) 5 d cocultivated leaf segments showing blue spots in directly infected (left) and sonicated (right) leaf explants.

Supplementary material Fig. S2



PCR analysis of the T_1 progeny raised from L1 transgenic line. Out of 20 plants 14 plants were PCR positive which gave an approximate Mendelian ratio of 3:1.



PCR analysis of the T_1 progeny raised from L2 transgenic line. Out of 18 plants 12 plants were PCR positive which gave an approximate Mendelian ratio of 3:1.

Table S1 Effect of explant source on survival of leaf explants, and transient transformation efficiency 5 d after cocultivation

*Explant source	Percentage of bacterial/fungal contamination ± S.E.	Surviving explants after cocultivation (Mean ± S.E.)	** Explants (%) expressing Gus	***Regenerating explants (%) (Mean ± S.E.)
In vitro-shoots	0	62.3 ± 1.5	33.3 ± 1.0	30.0 ± 1.2
GH-grown seedlings	23.7 ± 1.9	84.3 ± 1.2	70.5 ± 1.5	62.7 ± 1.8

*120 leaf explants were cocultivated from each source
**10 explants for histochemical Gus assay
***Response in respect of callus associated with shoot regeneration

Table S2 Expression of β-glucuronidase in leaves collected from different positions in T_0 transgenic plants of *W. somnifera*

Transgenic plant	Gus activity in leaf (nmol/min/mg protein) ± S.E.		
	Upper	Middle	Lower
L1	260.57 ± 2.4	96.48 ± 2.5	66.42 ± 2.6
L2	206.35 ± 2.8	107.24 ± 1.8	62.65 ± 1.6