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Agrobacterium-mediated transient transformation of *Pentalinon andrieuxii* Müll. Arg.

Alejandro Yam-Puc[1], Elidé Avilés-Berzunza[1,2], Manuel J. Chan-Bacab[3], Luis Manuel Peña-Rodríguez[1], Gregorio Godoy-Hernández[1,2*]

[1]Centro de Investigación Científica de Yucatán, A.C. Laboratorio de Química Orgánica, Unidad de Biotecnología, Mérida, México
[2]Unidad de Bioquímica y Biología Molecular, Mérida, México
[3]Departamento de Microbiología Ambiental y Biotecnología, Universidad Autónoma de Campeche, Campeche, México
Email: [*]ggodoy@cicy.mx, alexqbb@cicy.mx, avibeli@cicy.mx, manjchan@mail.uacam.mx, lmanuel@cicy.mx

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ABSTRACT

Sections of hypocotyls, roots and leaves from *Pentalinon andrieuxii* plantlets were transiently transformed with *Agrobacterium tumefaciens* LBA4404 bearing the binary plasmid pCAMBIA2301 with an interrupted *β*-glucuronidase (GUS) gene. Histochemical GUS assays showed transient gene expression in all infected tissues, being older roots those which displayed the most intense GUS staining. To our knowledge, this is the first report of *Pentalinon andrieuxii* susceptibility to *Agrobacterium tumefaciens*-mediated genetic transformation.

Keywords: *β*-Glucuronidase; *Agrobacterium tumefaciens*; Kanamycin; *Apocynaceae*

1. INTRODUCTION

Pentalinon andrieuxii Müll. Arg. (*Apocynaceae*), a plant commonly named "contrayerba" in the Yucatan Peninsula, is used in the treatment of *Leishmania*'s skin lesions ("chiclero's ulcer"). Mayan healers also recommend chewing their roots and leaves to relieve ailments derived from snakebites, and the stem-collected latex to alleviate headaches and nervous disturbances [1,2]. Cardenolides, pyrrolizidine alkaloids, steroidal compounds and betulinic acid derivatives with different physiological activities have been found in *Pentalinon* tissues [3-6]. Furthermore, two physiologically inactive, but structurally unusual trinorsesquiterpenoids, named urechitols A and B have been described in this species. Urechitols include the novel bicyclic *campechane* skeleton, which is formed by two cyclic nuclei of five and seven carbon units, respectively [7]. Although, the synthesis of racemic mixtures of urechitol A was recently reported [8], the biosynthetic origin of urechitols or the *campechane* skeleton remains unknown. The availability of *in vitro* culture systems of *P. andrieuxii* tissues, including those used for genetic transformation, may allow not only the controlled production of these compounds, but also the development of the basic tools for functional genetics applied to the identification of genes involved in the biosynthetic pathway of urechitols. In here, we report the development of a protocol for the transient transformation of *P. andrieuxii* explants with *Agrobacterium tumefaciens*.

2. MATERIALS AND METHODS

2.1. Plant Material

P. andrieuxii seeds were collected from mature dehiscent siliques in February 2009 from a population located 3.5 km northeast from Campeche City, Mexico (19˚51'0"N, 90˚31'50"W). A voucher specimen was deposited in the Herbarium of Centro de Investigación Científica de Yucatán (P. Sima 2503). Seeds were surface sterilized with 5% Extran and 70% ethanol for 5 min each, followed by immersion in a 50% bleach solution (3% sodium hypochlorite) for 20 min. Disinfested seeds were germinated in modified hormone-free PC-L2 medium [9] pH 5.5, supplemented with 2.5% sucrose and 1% agar. Eight seeds per container were incubated at 25˚C ± 2˚C for two weeks in the dark, and then on, under continuous light (40 - 50 mmol·m^2·seg^{-1}).

2.2. *Agrobacterium tumefaciens* Strains and Vectors

Agrobacterium tumefaciens LBA4404 strain and the binary vector pCAMBIA 2301 (Center for the Applications of Molecular Biology to International Agriculture, Canberra, Australia (http://www.cambia.org/daisy/bioforge_legacy/3724.html)) were used in all experiments. The pCAMBIA 2301 plasmid contains the neomycin phosphotransferase II (*nptII*) gene for kanamycin selection, in

[*]Corresponding author.

addition to the *uidA* gene encoding the *β*-glucuronidase gene (GUS), interrupted by the catalase intron, for the phenotypical selection of transformed tissues. Both genetic markers are driven by the CaMV 35S promotor. The presence of an intron in *uidA* ensures expression in eukaryotic cells. *A. tumefaciens* was cultured in liquid yeast extract and beef (YEB) medium, pH 5.6, containing 100 mg·l^{-1} rifampicin and streptomycin each (Sigma, St. Louis, MO). Cultures were kept in the dark at 28˚C for 48 h. Bacteria were made competent with $CaCl_2$ and transformed with the plasmid *via* heat shock [10]. Transformed cells, harboring pCAMBIA 2301, were screened on semisolid YEB media with 100 mg·l^{-1} rifampicin and streptomycin each, and 50 mg·l^{-1} kanamycin (YEB-AB), and then, cultured in 10 ml of liquid YEB-AB medium (pH 5.6). Cultures were kept at 28˚C for 48 h in a rotatory shaker (200 rpm). A 200-μl aliquot of this suspension was diluted in 10 ml of YEB-AB, and further incubated for 24 h as described. Culture volume was then completed to 20 ml with YEB-AB, and added with 200 μM acetosyrin-gone (AS). This was incubated up to 5 h, prior to tissue inoculation.

2.3. Genetic Transformation

Sections of hypocotyls, roots (10 mm length) and leaves (*ca*. 0.25 cm^2) were excised from 15 day-old plantlets and superficially wounded in a longitudinal manner with a scalpel prior to infection with *A. tumefaciens*. Explants were vacuum infiltrated with a 20 ml of a 0.1 OD_{600} bacterial suspension in PC-L2 medium for 20 min. Tissues were then blotted with sterile filter paper in order to eliminate the bacterial excess, and placed on semisolid PC-L2 medium for 72 h. After 3 days of cocultivation, at 28˚C in the dark, explants were transferred to semisolid PC-L2 medium supplemented with 100 mg·l^{-1} cefotaxime and 10 mg·l^{-1} kanamycin for further development. Transient GUS expression was histochemically assayed 3, 6 and 21 days after infection by staining transformed tissues with 5-bromo-4-chloro-3-indolyl-*β*-D-glucuronide (X-GLUC) [11]. Briefly, 50 explants were vacuum-infiltrated in the buffer solution for 5 min, and thereafter incubated for 24 h at 37˚C in the dark [12]. GUS activity was estimated by the number of blue spots per explant after washing them in a 3:1 (v/v) mixture of methanol: acetone. A blue spot was considered as a single transient GUS-expression focus.

3. RESULTS AND DISCUSSION

Transient Transformation of *Pentalinon andrieuxii*

After 21 days of infection, transient GUS expression was found in both root and leaf sections (**Figure 1**). Positive GUS activity could be solely attributed to the expression and correct splicing of the inserted *uidA* in eukaryotic plant cells. *P. andrieuxii* does not present endogenous GUS activity as reported for carrot, celery, parsley, rice [13], *Ricinus communis* [14], *Agave fourcroydes* [15] and *Capsicum chinense* [16]. Older roots displayed a higher GUS activity than younger root explants and leaf sections. Interestingly, even when hypocotyls' explants did not show GUS activity during the first 20 days of infection, after a total of 40 days and once tissues had turned into undifferentiated calli, significant activity was observed (**Figure 1**). Older root tissues (two months) were the most susceptible to infection with a 26% of positive transformation events, followed by leaves (15%) and younger roots (3.3%) (**Table 1**). Furthermore, older roots also presented the highest foci number per explant in comparison to leaf sections (7.83 vs 3.96).

4. CONCLUSION

In conclusion, we have developed the first protocol for transient genetic transformation of *P. andrieuxii.* At the present time, this method is being used to probe a protocol for its stable genetic transformation, which in combination with the already developed plant regeneration system [17], will allow to assay the functional role of genes putatively involved in secondary metabolism.

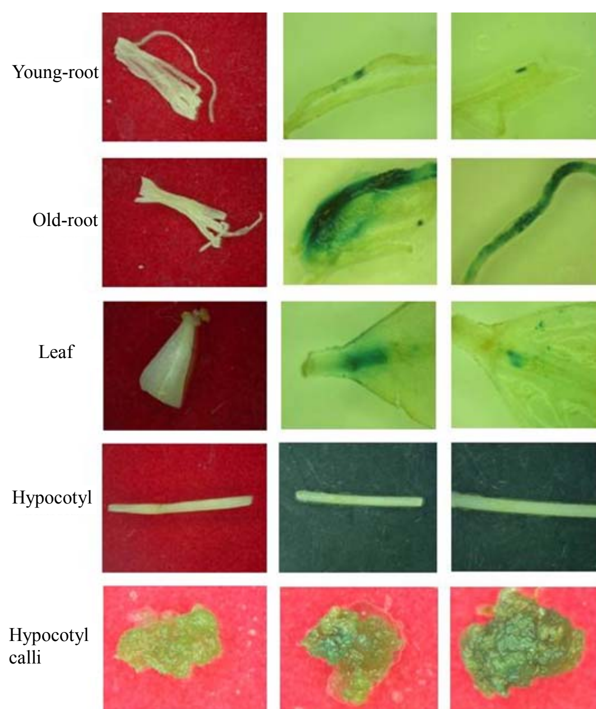

Figure 1. Histochemical GUS assay performed on tansformed explants of *Pentalinon andrieuxii* Müll. Arg. Explants tansformed with Pcambia2301 via *Agrobacterium tumefaciens* stain LBA4404.

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Table 1. Infection frequency and number of GUS foci in *P. andrieuxii* explants.

Explants	Explant number	Number of GUS positive explants	Infection frequency (%)[1]	Number of GUS foci/explants[2,3]
Young-root	450	15	3.33	1.00 (0)[c]
Old-root	300	78	26.00	7.83 (3.5)[a]
Leaf	360	54	15.00	3.96 (0.6)[ab]
Hypocotyl	360	0	0	0

Histochemical analysis assays were performed on explants twenty one days after inoculation with *Agrobacterium tumefaciens* strain LBA 4404: pCAMBIA2301. [1]Infection frequency (%) = number of GUS positive explants/ total number of infected explants × 100. [2]Number of GUS foci/explants, was the average of GUS positive foci in at least three independent experiments with more of 100 explants each one, with standard deviation in brackets. [3]Values with different letters are statiscally different (P = 0.05) according to Tukey's test.

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