

***Agrobacterium*-mediated transient transformation of Mexican prickly poppy (*Argemone mexicana* L.)**

Gregorio Godoy-Hernández*

Unidad de Bioquímica y Biología Molecular de Plantas
Centro de Investigación Científica de Yucatán
A.C. C. 43 No. 130, Chuburná de Hidalgo 97200
Mérida, Yucatán, México
Tel: 999 9428330
Fax: 999 9 81 39 00
E-mail: ggodoy@cicy.mx

Elidé Avilés-Berzunza

Unidad de Bioquímica y Biología Molecular de Plantas
Centro de Investigación Científica de Yucatán
A.C. C. 43 No. 130, Chuburná de Hidalgo 97200
Mérida, Yucatán, México
Tel: 999 9428330
Fax: 999 9 81 39 00
E-mail: avibeli@cicy.mx

Mildred Carrillo-Pech

Unidad de Bioquímica y Biología Molecular de Plantas
Centro de Investigación Científica de Yucatán
A.C. C. 43 No. 130, Chuburná de Hidalgo 97200
Mérida, Yucatán, México
Tel: 999 9428330
Fax: 999 9 81 39 00
E-mail: mild@cicy.mx

Felipe Vázquez-Flota

Unidad de Bioquímica y Biología Molecular de Plantas
Centro de Investigación Científica de Yucatán
A.C. C. 43 No. 130, Chuburná de Hidalgo 97200
Mérida, Yucatán, México
Tel: 999 9428330
Fax: 999 9 81 39 00
E-mail: felipe@cicy.mx

Website: <http://www.cicy.mx>

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Abbreviations: AS: acetosyringone
GUS: β -glucuronidase = EC. 3.2.1.31
TDZ: Thidiazuron = 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea
YEB: Yeast Extract and Beef

Shoot apex, leaf primordia, leaf sections and roots from Mexican prickly poppy seedlings, were inoculated with *Agrobacterium tumefaciens* harboring the binary vector pCAMBIA2301, which contained the β -glucuronidase (*uid A*) gene. Histochemical β -glucuronidase (GUS) assay in infected explants showed transient *gus* gene expression between 3 and 12 days after inoculation. To our knowledge, this is the first report of *A. mexicana* susceptibility to *A. tumefaciens*-mediated genetic transformation.

The genus *Argemone* (Papaveraceae) comprises almost 30 species, all with prickly stems, leaves and capsules. *Argemone mexicana* L., commonly known as Mexican or prickly poppy, is native to tropical America, but has widely spread to many tropical and sub-tropical regions. It is frequently found as a weed in cultivated and abandoned fields. It is an annual, hermaphroditic shrub, mostly pollinated by insects, with terminal bright yellow flowers (Rao and Dave, 2001). In Mexican traditional medicine,

*Corresponding author

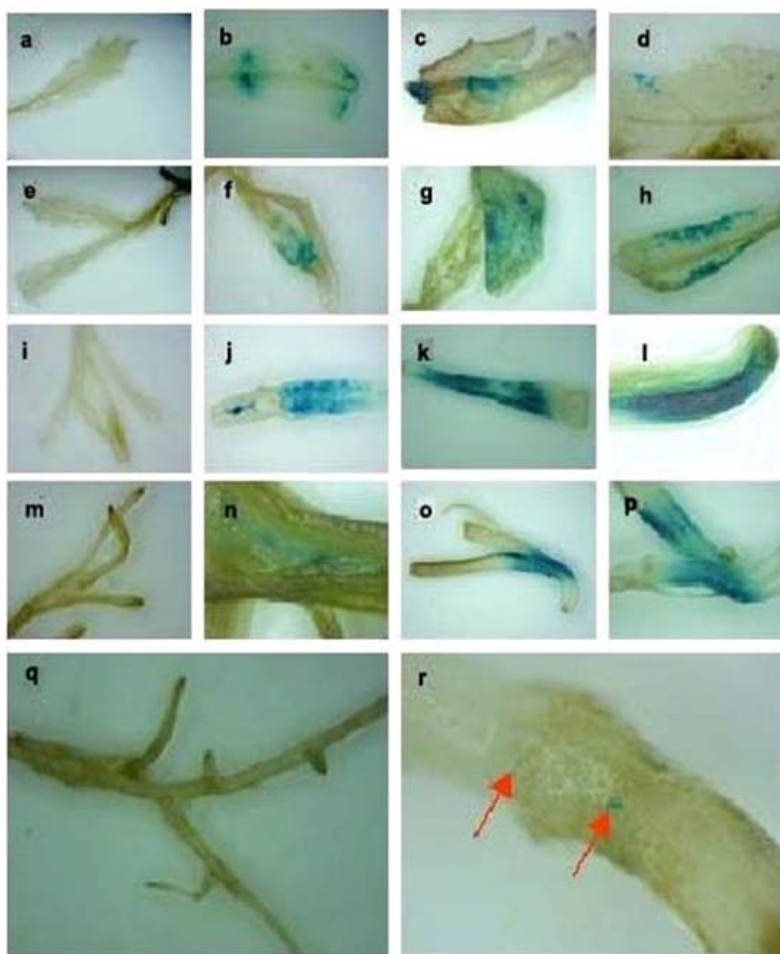


Figure 1. Histochemical GUS assays performed on transformed explants of *Argemone mexicana* L. Explants were transformed with the binary vector pCAMBIA2301 via *Agrobacterium tumefaciens* (strain LBA4404). (a), (e), (i), (m) and (q), negative, untransformed controls; (b), (c), (d), (f), (g), (h), (j), (k) and (l), transiently transformed leaves; (n), (o) and (p), transiently transformed shoot-apex sections; (r): transiently transformed roots. The arrows point to GUS expression foci.

Argemone plants have been used to treat different skin and eye ailments.

Seeds are rich in non saturated fatty acids, such as linoleic, oleic, ricinoleic, and palmitoleic acids. Nevertheless, their high content of toxic compounds may hamper their use with industrial purposes. In fact, the presence of *Argemone* oil in edible mustard oil is probably responsible for outbreaks of epidemic dropsy, a clinical syndrome characterized by edema, cardiac insufficiency, renal failure and glaucoma, which is attributed to sanguinarine, the main *Argemone* alkaloid (García et al. 2006). Sanguinarine is a quaternary benzophenanthridine alkaloid with a strong bactericidal effect on Gram-positive bacteria (Rao and Dave, 2001). It also displays anti-inflammatory and cytotoxic activities. The antimicrobial and physiological effects of sanguinarine suggest that it may confer protection against diverse pathogens (Facchini, 2001).

Other identified alkaloids in *Argemone* include berberine, protopine and coptisine (Facchini, 2001). The complex alkaloid mixture of this plant is perhaps the basis for its allelopathic, nematocidal and bactericidal effects. In this way, *A. mexicana* has many potential uses, some of them derived of the presence of alkaloids, while others may be handicapped by these same toxic compounds. In any case, the development of tools for the genetic improvement of this plant is required, either to promote or to reduce alkaloid accumulation. Genetic improvement of *A. mexicana* by conventional breeding may prove difficult, since it is a non-domesticated specie with a narrow genetic variability within the available germplasm. Moreover, it also presents strong seed dormancy. An alternative to overcome these limitations is the introduction of new traits by *Agrobacterium*-mediated genetic transformation, which remains as the most employed method for transferring genes into plants cells. We are reporting transient

β -glucuronidase (GUS) expression in prickly poppy via *Agrobacterium tumefaciens* transformation. To our knowledge, this is the first report of *A. mexicana* susceptibility to *A. tumefaciens*-mediated genetic transformation.

MATERIALS AND METHODS

Plants of *Argemone mexicana* were collected in the outskirts of Mérida, México (20°58'00"N/89°37'00"W) and taxonomically identified by Dr. Roger Orellana-Lanza (Regional Botanical Garden, Centro de Investigación Científica de Yucatán, México). One voucher has been deposited at the CICY herbarium (F Vázquez-Flota and R Orellana 01). Seeds were collected from mature, dehiscent capsules and soaked for 2 hrs in 10% sulfuric acid, followed by subsequent washes in 70% ethanol (30 sec), sodium hypochlorite 0.6% (10 min) and sterile distilled water. Seeds were then plated on G medium, prepared with the Phillips and Collins (1979) salts at half ionic strength, without growth regulators (pH 5.5) and incubated at 25 ± 2°C under continuous light (photon flux density of 40-60 $\mu\text{mol m}^{-2} \text{s}^{-1}$), provided by fluorescent lamps (39 W; Philips, USA).

A. tumefaciens strain LBA4404 was used in all experiments. Binary vector pCAMBIA2301 (Center for the Application of Molecular Biology to International Agriculture, Canberra, Australia), containing the β -glucuronidase (*uid A*) and the neomycin phosphotransferase II (*npt*) genes within the T-DNA borders. The *uid A* gene in pCAMBIA2301 is interrupted by a catalase intron, which has to be removed for eukaryotic expression. Bacteria were cultured at 28°C in the dark in liquid YEB (Yeast Extract and Beef) medium at pH 7, supplemented with 100 mg l⁻¹ rifampicin and 100 mg l⁻¹ streptomycin (YEB is 5 g l⁻¹ of each beef extract, peptone and sucrose, 1 g l⁻¹ yeast extract, and 0.5 g l⁻¹ MgSO₄·7H₂O). Bacteria were made competent

with CaCl₂ and transformed with plasmids by heat shock (Zhang and Zeevaart, 1999). Transformed cells, harboring pCAMBIA2301, were screened on semisolid (15 g l⁻¹ bacto-agar) YEB medium, containing 100 mg l⁻¹ rifampicin, 100 mg l⁻¹ streptomycin and 50 mg l⁻¹ kanamycin, and then cultured in 10 ml of YEB liquid medium (pH 7) with the same antibiotics at 28°C for 48 hrs in a rotatory shaker (200 rpm). A 200 μl aliquote was then diluted in 10 ml of YEB, with the same antibiotic composition, and incubated for 24 hrs as described. Culture volume was completed to a final volume of 20 ml with YEB, with the same antibiotic composition plus 200 μM de acetosyringone (AS), and further incubated up to 5 hrs, prior to tissue inoculation.

For the transformation experiments, shoot-apex, leaf sections and roots from six-week old prickly poppy seedlings (1-4 cm high) were taken and cut with a scalpel into 1 cm long strips. Explants were infected with a bacterial suspension, adjusted to 0.1 OD_{600 nm}, by immersion in 20 ml of I medium (I medium is PC-L2 liquid medium with 36 g l⁻¹ glucose, 68.4 g l⁻¹ sucrose, 100 mg l⁻¹ cysteine and 100 μM AS, pH 5.2), for 30 min. Tissues were blotted on sterilized filter paper to eliminate the bacterial excess, and then placed on CC medium (PC-L2 medium solidified with 8.0 g l⁻¹ agar, 30 g l⁻¹ sucrose, 100 mg l⁻¹ cysteine and 0.05 μM TDZ (Thidiazuron = 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea), pH 5.5). After 3 days of co cultivation at 28°C in the dark, explants were transferred to SF medium (PC-L2 medium supplemented 8.0 g l⁻¹ agar, 30 g l⁻¹ sucrose, 100 mg l⁻¹ cysteine, 0.05 μM TDZ, 100 mg l⁻¹ cefotaxime and 10 mg l⁻¹ kanamycin, pH 5.5). The cultures were maintained at 25 ± 2°C under continuous light (photon flux density of 40-60 $\mu\text{mol m}^{-2} \text{s}^{-1}$), provided by fluorescent lamps.

Transient expression of GUS was histochemically assayed from 3 to 12 days after infection by staining the explants with 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-

Table 1. Infection frequency in explants of *A. mexicana*. Histochemical GUS assays were performed on explants twelve days after inoculation with strain LBA4404::pCAMBIA2301.

Tissue	Infected explants	GUS positive explants	Frequency of infection ¹	GUS foci per explant ^{2,3}
Shoot apex	150	10	6.7	7.5 (3) ^a
Leaf primordia	150	9	6.0	17.0 (6) ^b
Leaves	150	6	4.0	19.3 (5) ^b
Roots	150	5	3.3	2.0 (1) ^a

¹Infection frequency was calculated as the percentage of GUS-positive explants per total number of explants examined.

²Number of GUS foci per explant was the average of GUS positive foci in at least three independent explants with standard deviation in brackets.

³Values with different letters are statistically different ($P = 0.05$), according to Tukey's test.

Gluc), according to Jefferson (1987). Briefly, approximately 50 explants were vacuum-infiltrated in the buffer solution for 5 min and thereafter incubated for 24 hrs at 37°C in the dark (Humara et al. 1999). Prior to counting the number of blue spots, explants were washed in methanol:acetone (3:1, v/v). Each blue spot was considered as a single transient GUS-expressing focus.

RESULTS AND DISCUSSION

Explants showed several blue spots produced by transient GUS expression 12 days after inoculation on leaves (Figure 1b, Figure 1c, Figure 1d, Figure 1f, and Figure 1g), leaf primordia (Figure 1h, Figure 1j, Figure 1k, and Figure 1l), shoot apex (Figure 1n, Figure 1o and Figure 1p) and roots (Figure 1r). In contrast, untransformed controls did not show any blue staining (Figure 1a, Figure 1e, Figure 1i, Figure 1m and Figure 1q). In roots, the LBA4404::pCAMBIA2301 vector resulted in a lower GUS expression, whereas in transformed shoot apex and leaf sections, blue staining was clearly visible.

GUS activity in tissues exposed to the bacteria can only be attributed to the actual expression of the *uid A* gene inserted into the plant genome, since it required the removal of the catalase intron. As a difference from other species, such as *Ricinus communis* (Rezmer et al. 1999) or *Agave fourcroydes* Lem., prickly poppy did not present endogenous GUS activity.

The infection frequency, evaluated as the number of GUS expression spots per transformed explant, was generally higher after 12 days rather than 3 days after infection. Leaf primordia, leaves and shoot apex sections presented the highest infection frequencies, while roots had the lowest ones (Table 1). As expected, the infection frequency was correlated to the seedlings' age. It is interesting to note that even when the frequency of infection was similar in shoot apexes and leaves, both primordial and mature ones, a statistically significant lower number of GUS expressing foci were detected on the former tissue. This observation suggests that leaves are more sensitive to *Agrobacterium* infection than shoot apex (Table 1).

Infection efficiency depends on several factors, including the procedure used to injure the tissues, bacterial density and co cultivation period (Humara et al. 1999). The highest infection efficiency for *Pinus pinea* cotyledons was obtained by combining sonication of tissues and the induction of bacterial virulence using AS (100 µM) 24 hrs prior to tissue incubation with an 1.0 OD_{600 nm} suspension for 72 hrs. However, explants developed necrosis after 30 days. Using a dilution (1:10) of bacterial suspension reduced tissue necrosis, but also shoot formation (Humara et al. 1999). In the work presented here, tissues were excised with a scalpel blade, and inoculation was performed by culturing explants with 0.1 OD_{600 nm} bacterial suspension for 72 hrs. Furthermore, a previous 5 hrs induction of bacterial virulence with AS was included. By

using this scheme, explants presented necrotic and yellowish areas that allowed shoot formation after 45-60 days. The longer survival period of tissues could be attributed to the lower bacterial density and to the incorporation of cysteine to the media, aimed to reduce tissue darkening, due to phenolization.

Actual GUS activity was not quantified since only transient transformation was achieved. Nevertheless, it is certain that tissues were genetically transformed given the fact that the catalase intron, which interrupts the *uid A* sequence in the vector (pCAMBIA2301), was removed during RNA processing in eukaryotic cells. Furthermore, *A. mexicana* tissues did not display endogenous Gus activity (Figure 1). To our knowledge, this is the first report of T-DNA transfer to *A. mexicana* tissues via *A. tumefaciens*. This procedure will allow us to introduce relevant genes in order to control of the synthesis of secondary metabolite through metabolic engineering (Samanani et al. 2002; Frick et al. 2004).

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