

Full Length Research Paper

***Agrobacterium*-mediated transient expression system in banana immature fruits**

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Transient expression assays are useful for saving time in studies of gene function, particularly ones expressed in fruit. Transient expression system of a reporter gene in immature fruit of banana was developed. *Agrobacterium tumefaciens*, whose plasmid contains an intron-containing β -glucuronidase (*gusA*) gene under regulatory control of the CaMV 35S promoter, was vacuum-infiltrated into sliced fruits and co-cultured. GUS histochemical assay was performed three days after co-cultivation, and a high level of GUS expression was observed. This transient expression system is useful for routine transient assays to validate genes expressed in banana fruit.

Key words: *Agrobacterium tumefaciens*, co-cultivation, GUS, *Musa*, promoter.

INTRODUCTION

After identifying thousands of promising gene sequences by genome projects, a quick analysis system is necessary to validate their functions in plants. Transgenic approaches have frequently been applied for this purpose. However, the production of stable transgenic plants is a lingering process, involving usually more than a year of regeneration, propagation and selection. In the case of banana, for example, successful genetic transformations were already reported using embryogenic cells or *in vitro* shoots (Sági et al., 1995; May et al., 1995; Becker et al., 2000; Ganapathi et al., 2001; Matsumoto et al., 2002; Pineda et al., 2002; Khanna et al., 2004; Tripathi et al., 2005; Huang et al., 2007; Ghosh et al., 2009). These methods generally needed a period of one to two years for establishment and plantlet regeneration. Additionally, the study of gene expression in fruits requires a further year for banana plant growth and fructification.

For gene function analysis, transient assays mediated by *Agrobacterium tumefaciens* have been increasingly applied as an alternative to stable transformation

(Johansen and Carrington, 2001; McIntosh et al., 2004; Wroblewski et al., 2005). In the present study, a transient gene expression system in banana immature fruits via *A. tumefaciens* is developed.

MATERIALS AND METHODS

Plant material

Immature fruits of triploid 'Nanicão' (*Musa* sp., AAA group, Cavendish subgroup) and diploid hybrid 'CNPMPF2803-01' (*Musa acuminata*, AA group) bananas were used in transient assays. Immature fruits, which fully developed and began to lose angularity but still maintained green color, were transversely cut into 20 to 30 mm pieces. Outer pieces were discarded, and inner ones were surface sterilized by 1% sodium hypochloride for 10 min and rinsed twice with sterile distilled water. For preventing tissue browning, the fruit pieces were sliced into 1 to 2 mm thickness immediately prior to *Agrobacterium* infection.

***Agrobacterium* and plasmid DNA**

Agrobacterium tumefaciens strain LBA4404 (from the collection of Embrapa Genetic Resources and Biotechnology, Brasília, Brazil) containing the binary plasmid pCAMBIA2301 (CAMBIA, Canberra, Australia) was used. The plasmid contains the neomycin phosphotransferase (*nptII*) coding region and intron-containing β -glucuronidase

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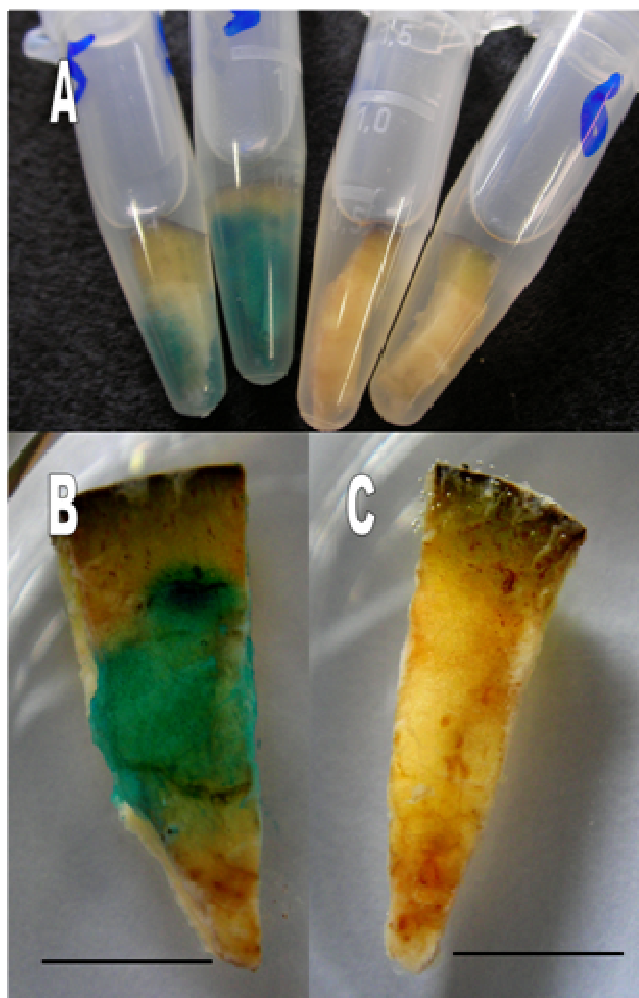


Figure 1. Transient GUS expression in immature fruit. (A) Sliced fruits were co-cultured with *Agrobacterium tumefaciens* with the binary plasmid pCambia2301 (left) and with no binary plasmid (right). (B) Detail of tissue co-cultured with the plasmid-containing bacteria. Scale bar = 3 mm. (C) Detail of tissue co-cultured with the plasmid-lacked bacteria. Scale bar = 3 mm.

dase (*gusA*) genes under regulatory control of the CaMV35S promoter. The *Agrobacterium* strain with no binary plasmid was used as a control for the experiments. The binary plasmid-containing bacteria were inoculated into solid MYA medium (Tepfer and Casse-Delbart, 1987) supplemented with 50 mg/l rifampicin, 300 mg/l streptomycin sulfate and 50 mg/l kanamycin, while the plasmid-lacked bacteria were inoculated into the same medium lacking kanamycin, at $26 \pm 1^\circ\text{C}$ for 2 days. An isolated colony of bacteria was inoculated into 20 ml of liquid MYA medium containing the same antibiotics and cultured on a horizontal shaker at 190 rpm and $26 \pm 1^\circ\text{C}$ until the bacterium cell density reached an OD_{600} of 0.8 - 1.0. An aliquot of 60 μl of the *Agrobacterium* cell suspension was again incubated in the same medium at same conditions for 2 days. After the period, the bacterium cells were collected by centrifugation at 8000 rpm (5900 g by rcf - relative centrifugal force) for 10 min and re-suspended to a OD_{600} of 0.4 in an infiltration medium (Rathjen et al., 1999) which consisted of 1/10 strength of MS salts

(Murashige and Skoog, 1962), 1/10 strength of B5 vitamins (Gamborg et al., 1968), 20 mM 4-morpholinepropanesulfonic acid (MOPS) (pH 5.4), 2% sucrose, 1% glucose and 200 μM acetosyringone.

***Agrobacterium* infiltration and co-cultivation**

Sliced fruits of 1 to 2 mm thickness were cut and divided into pieces of an eighth part. Each of two pieces were put into 1.5 ml microcentrifuge tubes containing one milliliter of the *Agrobacterium* cell suspension (OD_{600} 0.4) in the infiltration medium. They were placed in a floating tube rack at the center of a bath sonicator (Bransonic Ultrasonic Cleaner, 1210E-MTH) and sonication was applied for 0, 30 or 60 s. Then they were subjected to vacuum (630 - 660 mmHg) for 5 min, followed by maintaining a normal atmosphere in the dark for 25 min. Excess of the *Agrobacterium* containing medium was dropped out, and each of two fruit pieces were transferred to a well of 6-well plates (35 mm well-diameter, Corning - Sigma, USA) containing 2, 3 or 10 ml of co-cultivation medium. These volumes of medium were selected in such a way that the fruit pieces were immersed in half, intact, or above the depth in the medium, respectively. Three types of co-cultivation medium were tested: infiltration medium as described above; banana multiplication medium consisted of MS salts and vitamins, 22 μM benzylaminopurine, 3% sucrose and 200 μM acetosyringone (pH5.7); and banana regeneration medium consisted of 1/2 strength of MS salts, MS vitamins, 10 mg/l ascorbic acid, 10 mM L-proline, 3% sucrose and 200 μM acetosyringone (pH 5.8). The plates were maintained at $28 \pm 2^\circ\text{C}$ in the dark for 3 days. Each treatment was represented by 4 sliced tissues and repeated at least twice.

Histochemical GUS assay

The immature fruit pieces co-cultured with *Agrobacterium* for 3 days were transferred to 1.5ml microcentrifuge tubes containing 400 μl of X-gluc staining solution which consisted of 100 mM NaH_2PO_4 , 0.5 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 10 mM Na_2EDTA , 0.1% Triton®X-100, 1 mM X-gluc, pH7.0 (Jefferson, 1987). The tubes were subjected to vacuum (630 - 660 mmHg) for 5 min and incubated for 24 h at 37°C in the dark. After rinsing in 70% ethanol, GUS activity shown as blue color staining of the fruit pieces was captured as digitalized photo images of JPEG format. The relation of GUS expressing area to total tissue area was measured and calculated using ImageJ® computer program (<http://rsbweb.nih.gov/ij>; Wayne Rasband (wayne@codon.nih.gov), the Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA.).

RESULTS AND DISCUSSION

Immature fruit pieces co-cultured with *A. tumefaciens* containing pCambia2301 showed a high level of transient GUS expression (Figure 1). When they were co-cultured with *Agrobacterium* strain with no binary plasmid (control), no GUS expression was observed. There was a slight difference in the expression level between banana varieties of 'Nanicão' (AAA) and 'CNPMF2803-01' (AA) when sonication was not applied (Table 1). Sonication did not show influence in 'CNPMF2803-01' but shows negative influence in 'Nanicão'. The wounding of target tissues by sonication increased transient GUS expression in

Table 1. Effect of variety and sonication time on transient GUS expression in immature fruit slices mediated by the percentage of expressed area in total tissue area¹.

Variety	Sonication time (s)		
	0	30	60
Nanicão	42.6 ± 3.0Aa ²	31.1 ± 3.5Ab	30.2 ± 1.6Ab
CNPMF2803	24.0 ± 2.3Ba	27.7 ± 7.3Aa	39.2 ± 12.6Aa

¹After sonication, sliced tissues were co-cultured in 2 ml infiltration medium with the pCAMBIA2301 containing *Agrobacterium tumefaciens*.

²Mean value ± standard deviation. Means followed by the same capital letter in the same column and the same lower case letter in the same line do not differ according to the Tukey test ($p < 0.05$).

Table 2. Effect of co-cultivation medium on transient GUS expression in immature fruit slices of 'CNPMF2803' mediated by the percentage of expressed area in total tissue area¹.

Co-cultivation medium	GUS-expressed area (%)
Infiltration medium	31.6 ± 10.8 ²
Banana multiplication medium	0
Banana regeneration medium	0

¹Sliced tissues without sonication treatment were co-cultured in 2-ml co-cultivation medium with the pCAMBIA2301 containing *Agrobacterium tumefaciens*.

²Mean value ± standard deviation.

Table 3. Effect of volume of co-cultivation medium on transient GUS expression in immature fruit slices of 'Nanicão' mediated by the percentage of expressed area in total tissue area¹.

Volume of infiltration medium	GUS-expressed area (%)
2 ml	35.1 ± 6.6a ²
3 ml	4.9 ± 2.1b
10 ml	0b

¹Sliced tissues without sonication treatment were co-cultured in infiltration medium with the pCAMBIA2301 containing *Agrobacterium tumefaciens*.

²Mean value ± standard deviation. Means followed by the same letter in the same column do not differ according to the Tukey test ($p < 0.05$).

many species (Trick and Finer, 1997). However, it was not efficient on banana fruit slices (Table 1). It may be due to the fact that the sliced fruit tissues had been already sufficiently wounded. Therefore, further experiments were realized with no sonication. Conversely, co-cultivation medium and its volume had clear influence on

the transient expression (Table 2 and 3). The GUS expression was observed only on fruit tissues co-cultured in 2 ml of the infiltration medium. For stable transformation, co-cultivation media that stimulate growth of plant cells or tissues are commonly used (Ganapathi et al., 2001; Tripathi et al., 2005; Huang et al., 2007; Ghosh et al., 2009). Low transformation efficiency could be covered by high growth ratio and selection efficiency in plants. However, for transient expression assays, high transformation efficiency is essential. Co-cultivation medium that facilitates bacterium infection, such as the infiltration medium should be used to be able to induce sufficient gene expression. Additionally, the sliced tissues must not be immersed wholly in the co-cultivation medium, because the good aeration of plant tissues and bacteria is perhaps necessary for promoting transient gene expression. On the other hand, as preliminary experiments, a solid medium which makes good aeration was applied for co-cultivation, but GUS expression of the sliced tissues could not be observed because of its browning (Data was not shown).

In the above experiments, standard deviations of the mean value of GUS-expressed area (blue-stained area) were relatively high. The GUS-expressed areas differ from one repetition to the other. Thus, in promoter gene speculation, for example, two or more promoter genes similarly expressing may still be difficult to be compared by this system. More detailed studies, particularly in relation to fruit maturity must be realized. However, since more than 20% of blue-stained area were constantly obtained in diploid (CNPMF2803-01) and triploid (Nanicão) banana cultivars, the developed system seems to be useful for verifying whether one promoter gene functions in or not in fruit. In conclusion, the best transient GUS expression in fruit is obtained when immature fruit slices without sonication are half-immersed and co-cultured in liquid infiltration medium with a binary plasmid containing *A. tumefaciens*. This system could be useful for routine transient assays to validate genes expressed in banana fruits.

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