# Evaluación y selección de un protocolo vía *Agrobacterium* para la incorporación de resistencia al cogollero en la variedad de tomate Unapal-Arreboles

# Evaluation and selection of a protocol for *Agrobacterium*-mediated genetic transformation of tomato variety Unapal-Arreboles for resistance to budworm

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## RESUMEN

Se evaluó y seleccionó una metodología para la transformación genética de la variedad de tomate UNAPAL-Arreboles con el gen cry1Ab para la incorporación de resistencia al cogollero *(Tuta absoluta)*, utilizando el sistema de *Agrobacterium*. Se regeneraron 59 plantas transgénicas a partir de 3.200 explantes (1.84%). La integración estable, expresión y herencia de los genes nptII y gus-intrón, se demostraron mediante análisis histoquímico y molecular en los clones To28, To33 y To47 y en la correspondiente generación T1. Sin embargo, los análisis molecular e inmunológico indicaron ausencia del gen cry1Ab sugiriendo que la secuencia de este gen se puede haber modificado.

**Palabras clave:** *Solanum lycopersicon*; transformación genética; *Agrobacterium*; *Bacillus thuringiensis*; Cogollero; *Tuta absoluta*.

## ABSTRACT

A plant transformation methodology was selected and evaluated to incorporate the cry1Ab gene into the tomato variety UNAPAL-Arreboles for resistance to budworm (*Tuta absoluta*) using *Agrobacterium*-mediated genetic transformation. A total of 59 transgenic plants were regenerated from 3,200 explants (1.84%). Histochemical gus assay, molecular analysis of three independent events To28, To33 and To47, and corresponding T1-derived generations demonstrate the stable integration, expression and inheritance of the nptII and gus-intron genes. However, the molecular and immunological analysis of these same clones indicate that the cry1Ab gene is not present in the transformed plants, suggesting that the sequence of this gene may have been modified as result of possible recombinant events.

**Key words:** *Solanum lycopersicon*; Plant genetic transformation; *Agrobacterium*; *Bacillus thuringiensis*; budworm; T*uta absoluta*.

## INTRODUCTION

The budworm (*Tuta absoluta*), one of the most limiting pests in tomato production, is primarily controlled with pesticides, the indiscriminate use of which affects production costs, human and animal health, and the environment. The incorporation of resistance genes (present in related wild species) using conventional breeding is difficult due to genetic incompatibility between wild species and commercial varieties.

An alternative technology for introducing resistance genes is genetic transformation based on the use of the *cry* plasmid genes from *Bacillus thurigiensis* (Bt). These genes synthesize protein crystals in the middle intestine of the larvae, triggering the formation of non-specific pores, cellular lysis and death through septicemia (Lambert and Peferoen, 1992; Van Rie, *et al*., 1990).

Many *cry* genes have been identified and sequenced. Some have been modified and transferred for the control of insect pest larvae, such as cry1A for the pink bollworm that attacks cotton capsules (*Pectiniphora gossypiella)* (Wilson *et al*., 1992), cry3A for the Colorado potato beetle (*Leptinotarsa decemlineata*) (Perlak *et al*., 1993), cry1Ab for the European corn borer (*Ostrinia nubilalis)* (Koziel *et al*., 1993), cry1Ab in rice for the striped rice borer (*Chilo suppressalis*) and the rice leaffolder (*Cnaphalocrocis medinalis*) (Fujimoto *et al*., 1993), cry1Ab for the tobacco hornworm (*Manduca sexta*) in tomato (Fischhoff *et al*., 1987), and cry1Ab for the sugarcane borer (*Diatraea saccharalis*) (Arencibia *et al*., 1997).

Traits that have been introduced to tomato using genetic transformation include: resistance to kanamycin (Chyi et al., 1986; Koormeef et al., 1986; McCormick et al., 1986), tolerance to the herbicide glyphosate (Fillatti et al., 1987), tolerance to the herbicide Basta (De Block et al., 1987), resistance to the tobacco mosaic virus (Nelson et al., 1988), resistance to the alfalfa mosaic virus (Turner et al., 1987), resistance to the tomato spotted wilt virus (Gielen et al., 1996; Ultzen et al., 1995), resistance to insects (Vaeck et al., 1987; Fischhoff et al., 1987; Salm et al., 1994; Narváez-Vásquez, 1991), inhibition of resistance of the proteinase I and II inhibitors towards *Manduca sexta* (Orozco-Cárdenas, 1993), delay of fruit maturation (Smith et al., 1988; Hamilton et al., 1990), and fungal resistance (Yoder et al., 1988).

The success of genetic transformation of plants using *Agrobacterium* depends on the strain, the means of activation, the concentration of acetosyringone, the time of infection, the temperature and the time of co-culture. As these parameters need to be optimized in order to obtain efficient transformation of a particular variety, the object of this study was to evaluate and select a methodology for the genetic transformation of the tomato variety UNAPAL-Arreboles.

## MATERIALS AND METHODS

Preliminary tests were carried out in the International Center for Tropical Agriculture (CIAT). Three inactivated strains of *Agrobacterium* were used: Ag11 lacking the region T-DNA Mop (+), Cb (R); C58C1 region Mop=recA: bla (Lazo *et al*., 1991); and LBA4404, which carries the region *vir* of the plasmid ptiAch5 and lacks the region T-DNA of the plasmid Ti.

These strains, which conserve intact the region *vir* that is responsible for the excision, transport and integration of T-DNA from the bacterial genome to the plant cells, were transformed using electroporation with the plasmid pBIGCry (Cell-Porator ® Voltage Booster, Gibco –BRL). The 15.7Kb plasmid constructed in CIAT[1] contains a region of T-DNA modified with three chimeric genes (Figure 1). One of these genes codes for neomycin phosphotransferase II (*npt* II), which confers resistance to the aminoglycoside antibiotics used to select transformed tissue; and carried the promoter nos-5' and the termination sequence nos-3' of the gene nopaline synthase of *A. tumefaciens*.

The second *Cry*1Ab (3.0Kb), a modified version of the synthetic gene (Fujimoto *et al*., 1993) acquired by CIAT from the Japanese company Plantek, was introduced to the binary vector pBIGCry[1] together with the promoter 35S of the cauliflower mosaic virus (CaMV35S) and the termination sequence of the gene nopaline synthase (nos-3') of *A. tumefaciens.*

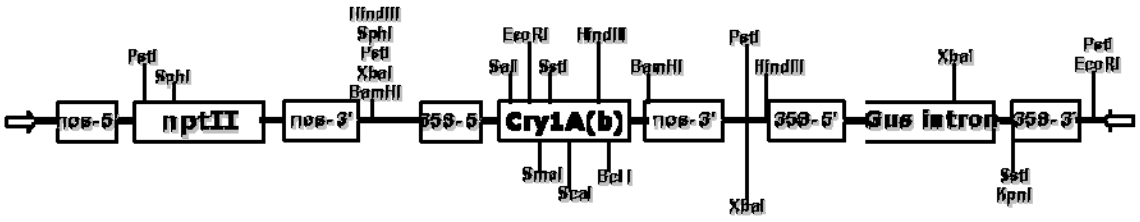


**Figure 1. Map of the T-DNA region of the pBIGCry plasmid, showing the chimeric genes nptII, cry1Ab and the gus-intron. (nos-5´ = promoter of nopaline synthase; nos-3´ = terminator of nopaline synthase; 35S-5´ = promoter of CaMV-35S and 35S-3´ = terminator of CaMV-35S).**
(Source: L. I. Mancilla. Personal Communication)

The third *gus*-intron codes for β-Glucuronidase, and allows the monitoring of transformed cells and tissues using a histochemical test that uses X-glu (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) as a substrate. In the presence of the enzyme, X-glu liberates an indigo blue color that stains the location of the gene expression in the tissue. This gene has as a promoter the region 35S-5' and the termination signal 35S-3' of the cauliflower mosaic virus.

In the exploratory test of bacterial activation three media conserved at -80$^{o}$C were used: MIB (Gelvin, 1989); PIM2 (Aldemita y Hodges, 1996) and Gelvin-Liu (Gelvin and Liu, 1994) (Table 1).

[1] Lida Inés Mancilla. 2000, Personal Communication. CIAT.

For MIB and Gelvin-Liu the following procedure was used: cultures of 5 ml were incubated for 16 hours in LB media (Sambrook, 1989) with selection antibiotics: kanamycin (50mgl$^{-1}$) and rifampicin (10mgl$^{-1}$) for Ag11/pBIGCry; kanamycin (50mgl$^{-1}$) and carbenicillin (100mgl$^{-1}$) for C58C1/pBIGCry; and kanamycin (50mgl$^{-1}$) and streptomycin (25mgl$^{-1}$) for LBA4404/pBIGCry. The cultures were centrifuged at 3000 rpm for 10 minutes and the precipitate was re-suspended in an equal volume with the activation media containing acetosyringone (AS) at a final concentration of 100 μM.

**Table 1. Composition of the bacterial activation media**

| Components | Gelvin-Liu | PIM2 | MIB |
|---|---|---|---|
| MES | 50mM | 75mM | 20mM |
| Glucose (%) | 0.5 | 1 | 0.5 |
| AB Salts (20X) | 1X | 1X | 1X |
| $NaH_2PO_4$ (mM) | 2 | 2 | 2 |
| AS (μM) | 100 | 100 | 100 |

For the procedure with PIM2, Petri dishes were inoculated with solid ABG medium (Gelvin and Liu, 1994), and incubated at 28 °C for three days. For the liquid culture, three tubes with 2 ml of YEP medium were inoculated simultaneously with antibiotics and a colony (Sambrook, 1989). The cultures were incubated at 28°C for 30 hours with constant agitation at 200 rpm. A pool of the three tubes was centrifuged at 3000 rpm for 15 minutes; the precipitates were re-suspended in 10 ml of the PIM2 medium containing 100 μM of AS, and were incubated at 28°C for 16 hours with constant agitation at 200 rpm. To estimate bacterial growth a spectrophotometer reading was made at 600 nm. When the culture presented an O.D. between 1.6 and 1.9, 20μl of AS (10 mgl$^{-1}$) was added, and the culture left on ice for an hour before infection.

For the infection with the media MIB and Gelvin-Liu, 25 explants with cotyledenous leaves were placed in Petri dishes with the medium M3. Five ml of the suspension was added at ambient temperature for 30 minutes. The explants were incubated upside down in the dark at 22$^{o}$C for 48 hours. The explant co-culture was then submitted to the gus test.

For the infection with the PIM2 medium, the explants were submerged in the suspension for 5 minutes, transferred to the medium M3 containing 100 μM of AS and a co-culture was made at 28$^{o}$C for 48 hours.

For the Gus test, the explants were submerged in X-glu and incubated for 16 hours at 37ºC. Those explants with positive expression presented blue points, especially at the edges where the cuts were made (Mendel *et al*., 1989).

## RESULTS AND DISCUSSION

### Selection of the bacterial strain

As the tests of transient expression of the *gus* gene did not show positive transformation of the Ag11 strain, the study used C58C1 and LBA4404. However, no significant differences were seen between these strains in the infection of explants in activated media. The differences were attributed to the composition of

each medium (Table 2, Figure 2). The highest values of transient expression were observed in the MIB medium, with infection with the strain LBA4404.

**Selection of the activation medium**

The expression of the *gus*-intron gene revealed that the best activation medium for the strain *Agrobacterium* LBA4404/pBI-GCry was MIB. This was attributed to the pH and glucose concentration of the medium (Figure 2).

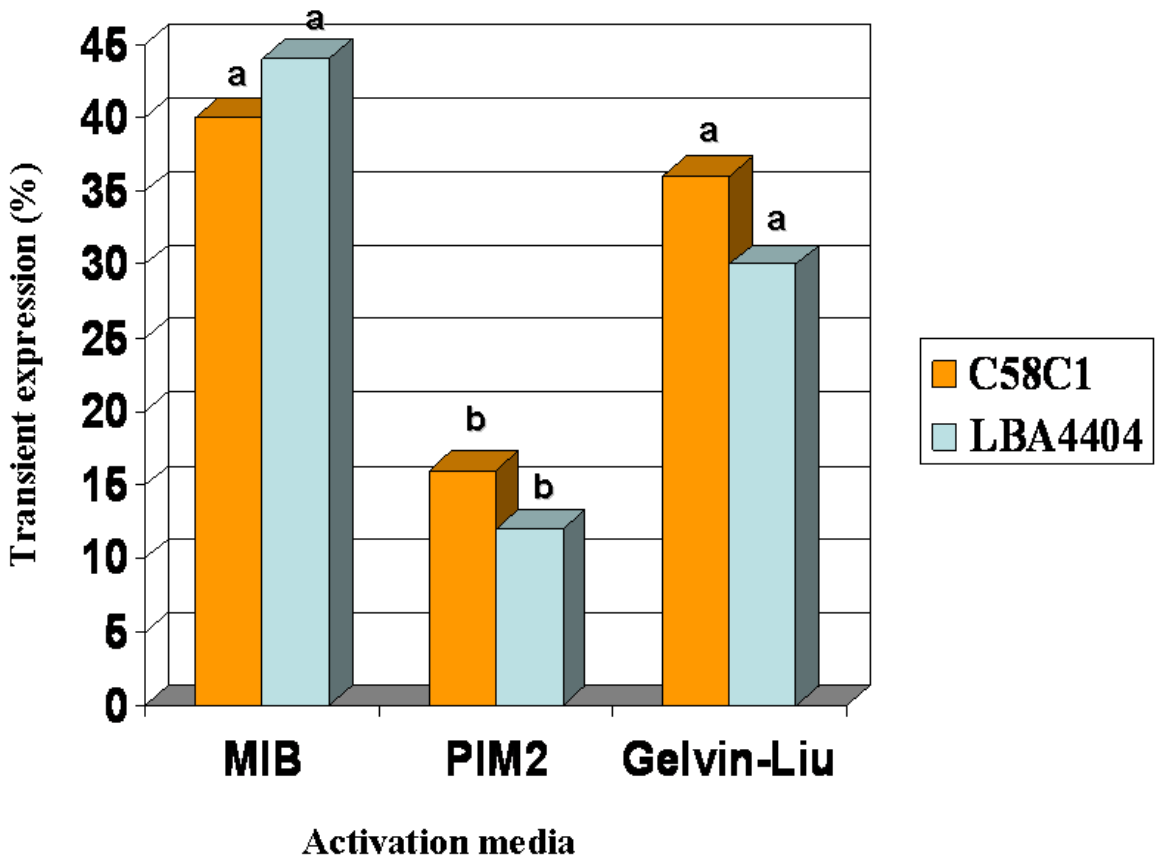


Figure 2. Transient expression of the gus-intron gene in explants of the tomato variety, UNAPAL-Arreboles infected with two strains of *Agrobacterium tumefaciens*, using three activation media.

Although there were no significant differences between the three infection times (30, 60 and 90 minutes), periods greater than 30 min could affect the transient expression by stressing the explants (Figure 3). Explant transformation success could depend on the duration of exposure to the inoculum, as long periods could produce high mortality through over-infection of bacteria, and over short durations transient expression may not be observed.

The bacterial concentration generally used for infection of tomato explants is approximately $5x10^8$ cells / ml (Fillatti *et al*., 1987, Narváez- Vasquez, 1991), or 5 x

$10^7$ cells / ml (Ultzen *et al.*, 1995) The strain LBA4404 in LB medium (3 ml) for 16 hours showed approximately this bacterial titre[2].

The transient expression of explants varied across the three concentrations of acetosyringone, but was not significantly different (Figure 4). The concentration of 100 μM of AS was adopted for the transformation protocol of the UNAPAL-Arreboles tomato variety.

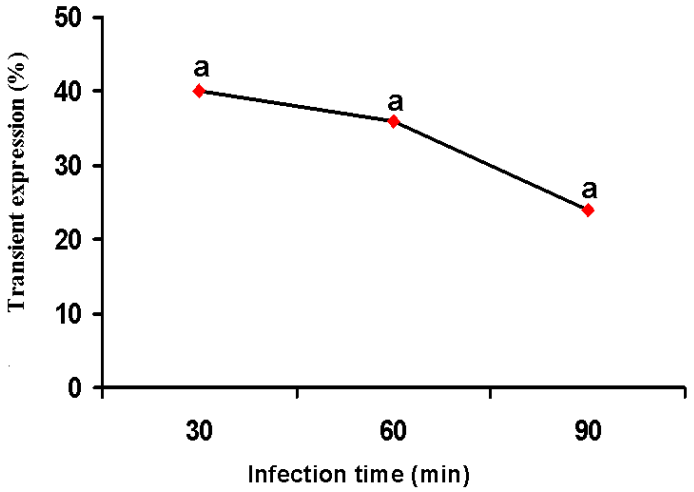


Figure 3. Effect of infection duration of the *Agrobacterium* strain LBA4404/pBIGCry on the transient expression of the gus-intron gene in cotyledenous explants of tomato, using the activation medium MIB. Differences are not statistically significant at p = 0.05.

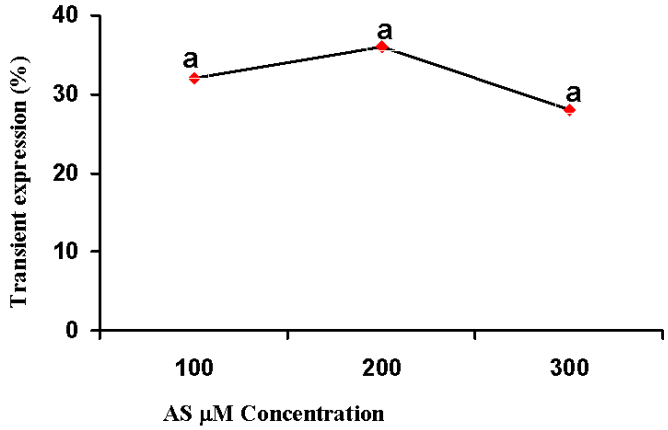


Figure 4. Effect of acetosyringone concentration in the activation medium MIB, on the transient expression of the gus-intron gene in tomato explants infected with the *Agrobacterium* strain LBA4404/pBIGCry. Differences are not statistically significant at p = 0.05.

There were no significant differences in the transient gene expression of the gus-intron between the co-culture temperatures of 22 and 25ºC, but there was between

[2] Sheila McCormick, 2001. Personal Communication. USDA

22 and 28ºC (Figure 5). This indicates that the optimum co-culture temperature of the strain LBA4404/pBIGCry and the UNAPAL-Arreboles tomato explant is 22ºC.

There were significant differences in transient expression between the durations of explant co-culture of 24 and 48 hours, and between 48 and 72 hours, but not between 24 and 72 hours (Figure 6). The low transient temperature at 24 hours could be due to it being an insufficient time for bacterial colonization, while with 72 hours, the excessive bacterial growth causes stress and tissue death. An optimum temperature of 48 hours is concordant with the results of Fillatti *et al*. (1987) and Ultzen *et al*. (1995).

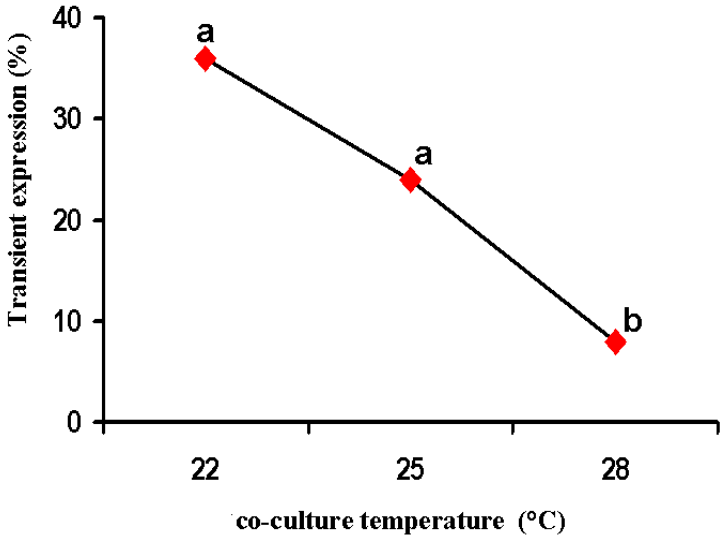


Figure 5. Effect of co-culture temperature on the transient expression of the gus-intron gene in tomato explants with the *Agrobacterium* strain LBA4404/pBIGCry. Values with different letters are significantly different at p = 0.05.

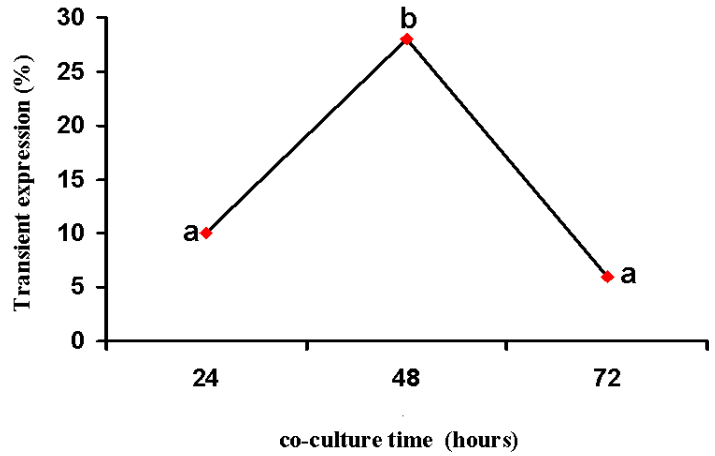


Figure 6. Effect of co-culture duration on the transient expression of the gus-intron gene of tomato explants infected with the *Agrobacterium* strain LBA4404/pBIGCry.
Values with different letters are significantly different at p = 0.05.

**Protocol for the transformation of the UNAPAL-Arreboles tomato variety**

Use the middle third of the cotyledenous leaves from plants of 7-10 days of age as explants. Place these in M3 medium with 50 explants per Petri dish (Figure 7a).

Infect the explants for 30 minutes with a 16 hour old culture of LBA4404/pBIGCry, activated with MIB containing 100 μM of acetosyringone (Figure 7b); Remove the bacteria and place the explants with the underside upwards; wrap the dishes in aluminum paper and place them in the dark at 22°c for 48 hours (Figure 7c); remove at random 10% of the explants for the gus test (Figure 7d), and place the rest in the selection medium (M3 with kanamycin 100 $mgl^{-1}$ and carbenicillin 500 $mgl^{-1}$), and incubate at 25-28°C with 16 hours of light / day, and illumination intensity of 80-100 μE $m^{-2}$ $s^{-1}$.

After three weeks, transfer the surviving explant shoots to M3 (zeatin is reduced to 0.1 $mgl^{-1}$ and AIA is eliminated), containing kanamycin (100 $mgl^{-1}$) and carbenicillin (500 $mgl^{-1}$). When the calluses produce shoots separate them and transfer them to fresh M3 with kanamycin 100 $mgl^{-1}$ and carbenicillin 500 $mgl^{-1}$ (Figure 7e).

Two or three weeks later cut the shoots with a height of more than 0.6 cm from the calluses, and place them in a rooting medium (1/4 M3 supplemented with 0.1 $mgl^{-1}$ ANA as a hormonal source, and 50 $mgl^{-1}$ of kanamycin) (Figure 7f).

When the plantlets have sufficient roots (Figure 7g) transfer them to sterile soil (Jiffy pots of 250 $cm^3$), and place them in a screened house. Cover them for five days with inverted polystyrene cups perforated at the base, and water them a little. Two weeks later fertilize them using 'Coljap desarrollo' (Figure 7h).

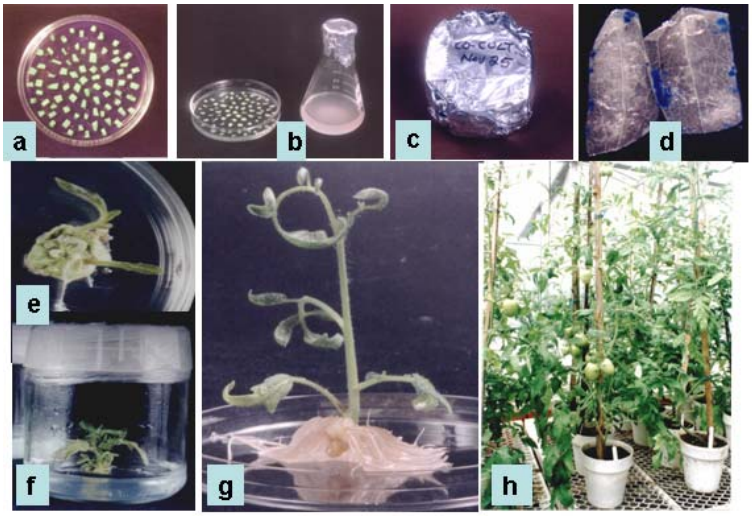


Figure 7. Stages of the protocol for transformation of the tomato variety UNAPAL-Arreboles. a. Explants from cotyledon leaves of 7-10 day old seedlings. b. Strain of *Agrobacterium* LBA4404 activated for explant infection. c. Explants infected in co-culture. d. Gus test for transformation efficiency. e. Shoot regeneration in selection medium. f. Plantlets in rooting medium. g. Rooted plantlet. h. Transformed plants in the biosafety screen house.

## Production de transformed tomatoes

From eight transformation events (400 explants per event) 59 kanamycin-resistant plantlets were isolated (1.84 % efficiency). Of these, eight clones did not root, six died in the growth chamber, and 37 presented a positive reaction in the gus test. In the screened house, 15 plants completed the vegetative cycle, producing 2-40 seeds per fruit and were vegetatively propagated from axillary buds. Of six clones that presented high expression of the gus-intron, three were chosen ($T_0$-28, $T_0$-33 and $T_0$-47) that showed typical bearing of the UNAPAL-Arreboles variety, better production of fruits (six per plant), easy propagation using axillary buds, and rapid development.

The biochemical analysis (kanamycin / gus test) of the clones T1-28, T1-33 and T1-47, and the molecular analysis of the clones To and T1, indicated the presence of the nptII and gus-intron genes in these transformed materials (Figures 8a and 8b). The immunological and molecular tests did not detect the cry1Ab gene, indicating its possible modification by recombination events or insertion of foreign DNA segments.

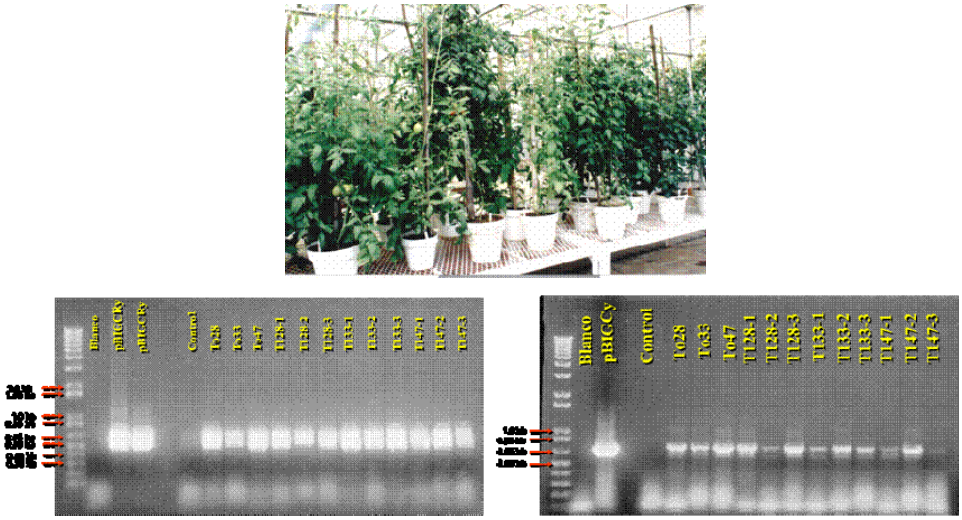


Figure 8. PCR analysis of plants To and T1 from transformed material (Clones T-28, T-33 and T-47). a. Transgenic plants To and T1 of the tomato variety UNAPAL-Arreboles. b. Detection of the presence of the nptII gene. c. Detection of the presence of the gus-intron intron. Line 1, Molecular Size Marker; Line 2, negative; Line 3, and 3 and 4, Plasmid pBIGcry; Line 5, Non-transgenic control; Lines 7-18 transformed tomato material.

## CONCLUSIONS

1. The medium MIB presented the highest values of transient expression when the explants were infected with LBA4404.

2. The optimal infection duration with LBA4404 was 30 minutes.

3. As the concentrations of acetosyringone (100, 200 and 300 μM) did not show significant differences in transient expression, the concentration of 100 μM was adopted in the transformation protocol.

4. The optimal temperature and duration for co-culture was 22°C and 48 hours.

5. The transgenic transformation efficiency for the UNAPAL-Arreboles tomato variety was 1.84%.

6. The integration and expression of the nptII and gus-intron genes was demonstrated both biochemically and molecularly in the first and second generations of the transformed tomato material.

7. Molecular and immunological analysis of the transformed clones did not detect the presence nor the expression of the cry1Ab gene.

## ACKNOWLEDGEMENTS

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