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Improving plant transformation using *Agrobacterium tumefaciens*

L.V. Ribeiro Neto, A.P. Oliveira, M.V. Lourenço, B.W. Bertoni, S.C. França, T.M. Rosa-Santos and S.M. Zingaretti

Laboratório de Genômica Funcional, Unidade de Biotecnologia, Universidade de Ribeirão Preto, Ribeirão Preto, SP, Brasil

Corresponding author: S.M. Zingaretti
E-mail: szingaretti@unaerp.br

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ABSTRACT. Here, we report a quick and low-cost method to improve plant transformation using *Agrobacterium tumefaciens*. This method involves the use of physical wounding, ultrasound, and an increase in exposure time to the bacteria. We show how the transformation rate increased from 0 to 14% when an ultrasound pulse of 10 s was used in conjunction with 96 h of bacterial exposure in *Eclipta alba* explants.

Key words: *Agrobacterium tumefaciens*; Plant transformation; Transgenesis

INTRODUCTION

Studies from the early 1970s determined that the source of tumor-inducing principle (TIP) in plants was the T-DNA portion of the Ti-plasmid from *Agrobacterium* spp. Because the Ti-plasmid could be used as a gene vector, the first transgenic plants became a reality (Stafford, 2000).

Several methods have been published, and transformation of plants, such as *Pelargonium* spp (García-Sogo et al., 2012), *Gossypium hirsutum* (L.) (Zhang et al., 2008) *Zea mays* (L.) (Ishida et al., 1996), and *Glycine max* (L.) Merrill (Olhoft et al., 2003), has been conducted using *A. tumefaciens*. However, the transformation frequency was always low, and the search for reliable and highly efficient methods has been a continuous objective. Several changes in important factors in the process of *Agrobacterium* transformation, such as acetosyringone concentration, co-cultivation periods, and temperature, are being tested for several plant species. It is also known that the bacterial binding and T-DNA integration can be a rate-limiting step, and the responses of plants to *Agrobacterium* strains can be different (Tie et al., 2012).

The aim of the current study was to evaluate the co-cultivation time required for and the effect of ultrasound pulse on the transformation of *Eclipta alba* with the *vip3A* gene from *Bacillus thuringiensis* cloned into pGA748 expression vector using *Agrobacterium tumefaciens* GV 3101 strain to increase the transformation rates.

MATERIAL AND METHODS

After initially being needle punched, *Eclipta alba* stem explants (2-cm long) were submitted to different ultrasound pulses of 40 kHz (0, 10, or 15 s) (Ultrasonic Cleaner, USC, 2800, Unique). Subsequently, the explants were transferred to 150-mL flasks that contained 20 mL Murashige and Skoog (MS) (Murashige and Skoog, 1992), 200 µM acetosyringone, and 100 µL transformed *A. tumefaciens* GV 3101 strain, which was previously grown to Optical Density 0.8 in YEB medium (Vervliet et al., 1975), supplemented with tetracycline (5.0 µg/mL), and kanamycin (50 µg/mL). Flasks were kept under 150 rpm agitation in the dark for different periods of time (0, 45, and 96 h) at 28°C. A total of 300 explants were distributed in 6 groups (50 explants) and identified as X (1-3) and T (1-3) and exposed to *Agrobacterium* for 45 or 96 h and ultrasound pulses of 0, 10, or 15 s.

After bacterial exposure, explants were transferred to solid MS medium supplemented with cefotaxime (250 mg/L). Flasks were kept at a constant temperature of 25° ± 1°C under daily white light/dark photoperiods (Ligh:Dark 16:8). The light source (Philips Linear Fluorescent, 046677410896, 40 W, T12, Cool White Premium) was maintained at a distance of approximately 5-10 cm from the flasks. Proof of transgenesis was performed by seedling selection in culture medium containing a lethal dose of kanamycin (500 mg/L) and by polymerase chain reaction (PCR). Genomic DNA was extracted using 40 mg of fresh tissue with Wizard® Genomic DNA Purification (PROMEGA-WI). A fragment of 2370 base pair was amplified using transgene specific primers (forward: 5'-ATG ACC AAG AAT AAT ACT AAA TTA AGC-3', and reverse: 5'-GAT CTT ACT TAA TAG AGA CAT GC-3'), in a polymerase chain reaction.

RESULTS

Initially, over 50% (161/300) of explants survived the transformation process and were transferred to MS medium containing kanamycin (500 mg/L) for the first selection. After

60-80 days, 46 explants (28.7%) were still viable. Survival under such restricted conditions indicate the presence of a kanamycin-resistance gene from the transgene insertion. For the second screening genomic DNA from all viable explants were amplified, however, PCR analysis confirmed the presence of the transgene in only 17 of the 46 survivors (36.9%) (Figure 1).

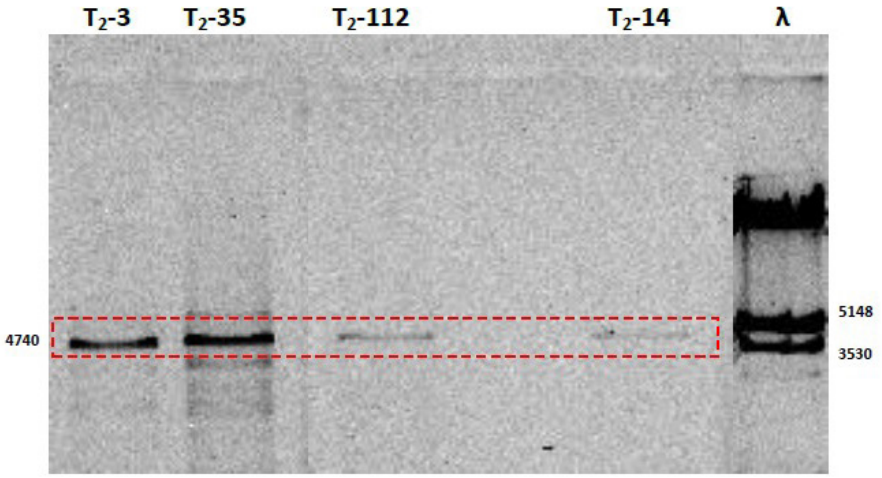

Figure 1. Proof of transgenes with PCR. Amplification reactions of some potential transgene explants using specific forward and reverse primers. Evidence shows that fragments of approximately 4740 bp are the result of double insertion and are present in some explants. The gel was run in 0.8% agarose in 1X TAE for 90 min at 100 V. λ *Eco*RI/*Hin*dIII molecular marker.

Except for the T1 group all groups had at least one transgenic explant (T3, X3). Groups manned T1toT3 present all together 8 transformants while in group X1 to X3 we were able to identify 8 transgenic explants. As can be seen in Table 1, explants from T3 and X3 group were subjected to ultrasonic pulses every 15 s, and although they survived for over 70 days, transgenesis only occurred in one explant of each group. In general, increasing co-cultivation time from 45 to 96 h had no effect in survival. The best gene transformation frequency (14%) was obtained with the combination of 10 s of ultrasound pulse and 96 h of explant exposure to the bacteria, which is an exceptionally high frequency for this gene transfer method.

Table 1. Transformation frequencies.

Code	Explants	Treatment	Transformants	Survival (days)	Transformation rate (%)
T1	50	96 h + 0 s	0	-	0
T2	50	96 h + 10 s	7	70-82	14
T3	50	96 h + 15 s	1	79	2
X1	50	45 h + 0 s	4	69-71	8
X2	50	45 h + 10 s	4	63-79	8
X3	50	45 h + 15 s	1	71	2

T1: explants not exposed to sonication and co-cultured for 96 h; T2: explants exposed to sonication for 10 s and co-cultured for 96 h co-culture; T3: explants exposed to sonication for 15 s and co-cultured for 96 h; X1: explants did not undergo ultrasound and co-cultured for 45 h; X2: explants exposed to sonication for 10 s and co-cultured for 45 h; X3: explants exposed to sonication for 15 s and co-cultured for 45 h; transformation rate was calculated by number of positive confirmations/total number of plants by treatment. h = hours of exposure; s = pulse time in seconds.

DISCUSSION

Taken together, our results indicate that increasing sonication time can be dangerous to plant tissues. Although relatively few transgenes were confirmed in explants (17 of 161, or 10.5%), this ratio of transformation is higher than that which is commonly observed using the *Agrobacterium* system and in transformation rates reported for *Begonia* x *hiemalis* Fotsch (0-3%) (Kishimoto et al., 2002); *Hordeum vulgare* (0-7%) (Shrawat et al., 2007), and an *Vigna unguiculata* (1%) (Chaudhury et al., 2007). However, considering the influence of ultrasound as well as time of contact with the bacteria, there was a significant increase in the number of transformed plants. The explants from the T2 group (96 h + 10 s) resulted in 7 transformants, or 14%, of the 50 explants assayed. The results obtained indicate that the use of ultrasonic pulses in conjunction with an appropriate exposure time to the transforming agent significantly increased the efficiency of transgenesis using *Agrobacterium tumefaciens*.

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