Factors influencing electroporation-mediated gene transfer to *Stylosanthes guianensis* (Aubl.) Sw. protoplasts

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Abstract

In order to develop a high-efficiency and reproducible transformation protocol for *Stylosanthes guianensis* we assessed the biological and physical parameters affecting plant electroporation protoplasts. Energy input, as combinations of electric field strengths discharged by different capacitors, electroporation buffer and DNA form were evaluated. Transformation efficiency was assayed *in vivo* as transient reporter gene expression, using the GFP-coding gene *mgfp5* driven by a CaMV 35S constitutive promoter. Energy input and electric field strength had a critical influence on transgene expression with higher transformation levels being achieved with 250 V.cm\(^{-1}\) discharged by 900 and 1000 µF capacitors. Linear plasmid DNA, the absence of chloride and the presence of calcium ions also increased transient gene expression, albeit not significantly.

Key words: direct gene transfer, plant transformation, forage legume, *Stylosanthes guianensis*.

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Introduction

The genus *Stylosanthes* (Fabaceae) comprises approximately 50 predominantly herbaceous species and subspecies native to tropical and subtropical regions of Asia, Africa and the Americas, principally South America. In the last two decades considerable progress has been made towards the identification of promising species for use as pasture in Brazil and Colombia (van der Stappen *et al*., 1998; Vieira *et al*., 1997; Liu *et al*., 1996).

Due to their vigorous growth habit, deep rooting ability and resistance to poor and infertile soils *Stylosanthes* species, associated with a wide range of grasses, are currently being used as protein banks, green manure and, principally, as forage crops (Lovato and Martins, 1997; Partridge, 1996). Although its characteristics favor the use of *Stylosanthes* as animal feed, the quantity and, especially, the quality of its proteins serve as constraints on its use as the sole feed source for extensive and intensive cattle feeding.

*Stylosanthes* is considered one of the least recalcitrant legume genus in respect to regeneration (Cônsoli *et al*., 1996; Dornelas *et al*., 1992; Dornelas *et al*., 1991; Meijer and Szabados, 1990; Vieira *et al*., 1990) and *in vitro* cell and tissue culture of *Stylosanthes* is well established, making molecular breeding strategies feasible (Portykus, 1990). The main drawback to long-term *in vitro* *Stylosanthes* culture is the induction of genetic and epigenetic changes (Cônsoli *et al*., 1996; Valarini *et al*., 1997).

To our knowledge, genetic transformation of *Stylosanthes* has only been mediated by the use of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* (Manners, 1987; Manners, 1988; Manners and Way, 1989) and transgenic plants have only recently been obtained (Sarria *et al*., 1994). These methods, depending on complex plant-pathogen interactions, result in low transformation efficiency due to legume-bacteria incompatibility (Ankenbauer and Nester, 1993; Cônsoli *et al*., 1995; Mauro *et al*., 1995; Yang, 1993) or selective marker negative effect on shoot regeneration (Hoffmann, 1998; Colby and Meredith, 1990).

Direct genetic transformation relies on physical and chemical forces to introduce foreign nucleic acid into a host genome, these methods having the advantage that they can be universally employed because no biological interaction is involved. Electroporation and microparticle bombardment are the principal systems for direct gene delivery to legumes (Aragão *et al*., 1999; Quorin *et al*., 1997; Portykus, 1991).

Biological membranes are composed of phospholipids, amphipatic molecules that have a hydrophilic head group attached to a hydrophobic tail, and are able to be polarized when submitted to electric fields. Electric pulses raise the transmembrane potential, promoting transient pore formation due to the increased dipole moment of the
hydrophilic lipid heads (Kinosita and Tsong, 1977; Neumann et al., 1982), allowing charged macromolecules to migrate through the pores and eventually reach the nucleus where they can promote genetic transformation.

Protoplast electroporation allows the introduction of foreign DNA into a great variety of cells, and these cells can be regenerated into transformed plants depending on the effectiveness of the shoot regeneration protocol. Under these conditions, transformation efficiency is higher than that obtained using high efficiency systems e.g. biolistics (Quecini, 1999).

In this article we describe a highly effective protocol for the transformation of *Stylosanthes guianensis* (Aubl.) Sw. (cv. Mineirão) protoplasts by electroporation. Electric field strength, energy input, DNA form and electroporation buffer were assessed in order to optimize transformation rates. Reporter gene expression and transgene PCR amplification was observed in regenerated plants.

**Material and Methods**

**Plant material and tissue culture methods**

Seeds of *Stylosanthes guianensis* (Aubl.) Sw. (cv. Mineirão) were provided by Embrapa/Cerrados (Brasília, Brasil). After surface sterilization (30 s in 70% ethanol followed by 15 min in 2% calcium hypochloride and abundant rinsing in autoclaved water) seeds were germinated *in vitro* in half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) without growth regulators but containing 1.5% (w/v) sucrose and solidified with 0.18% (w/v) Phytagel (Sigma). Fully expanded cotyledons excised from 7-8 day old seedlings were used for protoplast isolation as described by Vieira et al. (1990).

Freshly isolated protoplasts were re-suspended in electroporation buffer containing 10 μg of plasmid DNA per 800 μL (the volume of the electroporation cuvette) to give 2x10^6 protoplasts mL^-1 and the mixture gently agitated on ice for 30 min, after which the mixture was transferred to 0.4 cm electrode gap cuvettes (BioRad). The electroporation buffers investigated were designate buffer I (Fromm et al., 1987), buffer II (Tada et al., 1990) and buffer III (Walbot, 1993).

After electroporation, the protoplasts were carefully removed from the cuvettes and cultivated as described by Vieira et al. (1990) in liquid media (Kao, 1977 modified by Gilmour et al., 1989) at a density of 1x10^6 protoplasts mL^-1. Cultures were maintained under a 16 h light regime (75 ± 3 μE m^-2 s^-1) at 25 ± 2.4 °C. Shoots (2-4 cm long) were base excised from the callus and induced to root in MS medium containing 1% (w/v) sucrose, a regenerated plantlet being shown in Figure 1E. Individual shoots were

![Figure 1 - Plant regeneration from protoplasts of *Stylosanthes guianensis*. (A) isolated protoplasts 400 x, (B) *in vitro* dividing cell 400 x, (C) microcalli 200 x, (D) organogenic calli (bar =1.33 cm), (E) shoot initiation from calli (bar = 1.33 cm), (F) rooting plantlet (bar = 0.67 cm).](image-url)
histochemically assayed (Jefferson et al., 1987) for β-glucuronidase (GUS) expression. A specific polymerase chain reaction (PCR) transgene amplification method was also used.

Plasmid characteristics

The transient expression assay vector pCambia 1304 (CAMBIA GPO Box 3200, Canberra ACT 2601, Australia) carries a selective marker gene (hpt II) conferring hygromycin resistance and a fusion between the reporter genes coding for GUS (uidA) and a green fluorescent protein (GFP) (mgfp5), both driven by a 35S promoter from cauliflower mosaic virus (CaMV) as shown in Figure 2.

Plasmid DNA was independently transformed into E. coli JM 109 cells (Dower et al., 1988) or DH5a (Hanahan, 1983) and purified by the cesium chloride method (Sambrook et al., 1989). Supercoiled plasmid DNA was directly employed in transformation experiments or linearized by digestion with Eco RI (GIBCO BRL). Concentrations of plasmid DNA were spectrophotometrically determined at 260 nm and confirmed by Tris-borate EDTA (TBE)-gel electrophoresis in 0.8% agarose (Sambrook et al., 1989).

Electroporation conditions

Exponential decay pulses were applied by a commercial device (Bio Rad Gene Pulser II with Capacitance Extender) to 0.4 cm electrode gap cuvettes (800 µL) containing 1.6x10⁶ protoplasts and 10 µg of plasmid DNA.

Electric discharges from 400, 800, 900 and 1000 µF capacitors at electric field strengths of 125, 200, 250 and 300 V cm⁻¹ were used, providing the following energy inputs to the system: 500, 1280, 2000, 2880 mJ for the 400 µF capacitor; 1000, 2560, 4000 and 5760 mJ for the 800 µF capacitor; 1125, 2880, 4500 and 6480 mJ for the 900 µF capacitor; 1250, 3200, 5000 and 7200 mJ for the 1000 µF capacitor. Sample resistance was 20 Ω (buffers I and III) and 50 Ω (buffer II) as measured by the Gene Pulser. The length of the pulse (time) was established by the internal settings of the apparatus, and can be expressed as the pulse decay constant (τ = RC).

Analysis of GFP expression

Electroporated protoplasts were re-suspended in culture medium (Kao, 1977 modified by Gilmour et al., 1989) to give 1.0x10⁷ protoplasts mL⁻¹ and kept in the dark for 24 h at 4 °C. Non-electroporated protoplasts in 800 µL of electroporation buffer with 10 µg of plasmid DNA were ice-incubated for 45 min. As a measure of transformation efficiency, the number of transgene-expressing and non-expressing protoplasts were scored using fluorescence microscopy at λ = 395 nm.

Polymerase chain reaction (PCR) analysis

Genomic DNA was isolated from plantlets derived from electroporated or non-electroporated protoplasts as described by Edwards et al. (1991).

Each 25 µL of reaction mixture contained 20 ng of genomic DNA, 10 mM TRIS-HCl (pH 8.4), 50 mM KCl, 2.0 mM MgCl₂, 160 µM of each dNTP, 1.0 U of Taq polymerase (GIBCO BRL) and 200 nM of each uidA specific primer (gus 1 = CCT GTA GAA ACC CCA CAA CG and gus 2 = TGC AGC GCT ACC TAA GGC CG) (Figure 2), which provide an amplification product of 795 bp.

The mixture was overlaid with sterile mineral oil. Denaturation was performed at 95 °C for 2 min, followed by 25 amplification cycles in a Thermus (Perkin Elmer) thermal cycler (1 min at 94 °C, 1 min at 45 °C and 1.5 min at 72 °C) with a final extension of 7 min at 72 °C. DNA amplification products migrated on 1.4% (w/v) agarose TBE gels, were stained with ethidium bromide (10 µg mL⁻¹) and detected with ultraviolet light.

Figure 2 - Transformation vector pCambia 1304 expression cassette. PCR primers are indicated by (gus1) and (gus2). Arrows indicate cloning direction. Regulatory sequences (polyadenilation signals = polyA and promoters = pro) are shown as black segments. MCS = multicloning site, NOS = nopaline synthase, laczα = α subunit of bacterial lactase.
Results and Discussion

Optimizing the field strength for the delivery of foreign DNA to S. guianensis protoplasts

We used the transient expression of a gene coding for a green fluorescent protein (GFP) (Chalfie et al., 1994) for the in vivo evaluation of transformation efficiency. The frequency of transformants increased up to 3000 mJ of energy input (Figure 3A). While capacitors with a higher charge accumulation capacity (900 and 1000 µF) gave higher transformation frequencies (50%) when used in the range of 2000 to 4000 mJ, capacitors of lower charge accumulation (400 and 800 µF) led to the death of approximately 70% of the protoplasts (Figure 3A). Maximum transient gene expression has been reported under electric field strengths causing more than 50% reduction in protoplast viability (Fromm et al., 1985; Hauptmann et al., 1987; Oard et al., 1989).

Electric field (EF) strength is related to the electrode gap (d) and the discharged voltage (V) according to the equation \( EF = V.d^{-1} \), while the induced energy discharge (\( \varepsilon \)) is dependent on the capacitor and the applied voltage (V) according to \( \varepsilon = CV^2 \). Figure 3B shows that EF values of 250 V.cm\(^{-1}\) discharged by 900 and 1000 µF capacitors resulted in high transformation rates in electroporated protoplasts. Less intense electric fields were unable to promote reporter gene expression in more than 20% of electroporated protoplasts, while stronger electric fields induced irreparable plasma membrane damage, causing extensive cellular death and virtually no transformation.

Transient membrane pores are caused by an increase in the dipole moment of hydrophilic phospholipid heads, which move in the same direction as the applied electric field and provoke highly localized dielectric breakages in membrane structure (Kinosita and Tsong, 1977; Neumann et al., 1982; Neumann et al., 1996). Irreversible pores which lead to cellular death are caused by longer pulses and stronger electric fields (Langridge et al., 1987; Shillito et al., 1985), and we found that this occurred with energy inputs of more than 5000 mJ (Figure 3A) or electric fields stronger than 300 V.cm\(^{-1}\) (Figure 3B). Even so, protoplast electroporation has been reported as a mean of increasing cell division (Rech et al., 1987), plant regeneration (Chand et al., 1988; Ochatt et al., 1988) and DNA synthesis (Rech et al., 1988). However, in our experiments, plant regeneration was slower from electroporated than from non-electroporated protoplasts (Table I).

We observed specific transgene amplification in shoots regenerated from electroporated protoplasts (Figure 4).

Effect of the electroporation buffer on DNA introduction

Figure 5 shows that transient expression frequency of the GFP was significantly (p < 0.01) higher with buffer II and lowest with buffer I, while an intermediary frequency was obtained with buffer III. Non-viable and damaged protoplasts were rarely seen after electroporation in buffer II, which gave almost 100% expression of GFP (Figure 6A). However, we found that the frequency of stable

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<th>Table 1 - Comparison of plant regeneration efficiency from electroporated and non-electroporated S. guianensis protoplasts. See text for electroporation conditions.</th>
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\*Mean values of 4 independent experiments ± standard errors.
\(^1\) = 24 h after electroporation.
\(^2\) = N. of colonies after 45 days culture divided by the N. of plated protoplasts.
\(^3\) = N. of calli with shoots after 90 days of culture divided by the N. of plated calli.

Figure 3 - GFP expression in S. guianensis protoplasts electroporated with different electric forces. (A) energy input (mJ) and (B) electric field strength (V.cm\(^{-1}\)). Electroporation conditions were: 500 µF, 20 Ω, buffer I, 10 µg of supercoiled DNA.
transformation was lower than that expected from transient gene expression in electroporated protoplasts (Hain et al., 1985; Shillito et al., 1985), probably due to some retention of transgene expression ability in damaged protoplasts for periods shorter than 72 h. The chloride ions in buffer I resulted in the production of toxic chlorine gas when electric pulses were applied, leading to a reduction in cellular viability (Figure 6B). In contrast to the data reported by Senaratna et al. (1991), we found that non-electroporated protoplasts incubated with plasmid DNA under the same conditions for longer periods of time never showed transient expression of the reporter gene (Figure 6C).

In exponential wave electric circuits, the voltage decays exponentially in the electroporation buffer between the electrodes and the induced energy is dependent on the initially applied voltage and the charge accumulation capacity of the capacitor (Neumann et al., 1982, 1996). In this kind of circuit, the time constant (\(\tau\)) is a function of the electrical resistance (R) and the capacitance (C) of the circuit according to the equation \(\tau = RC\), so that for a given capacitor (C = constant) electroporation buffers with reduced ionic force impose less resistance to the movement of charge through the circuit and pulse decay time is longer. Kinosita and Tsong (1977) have demonstrated that longer duration pulses promote greater membrane permeability due to pore enlargement, and this probably contributed to the higher transformation rates which we observed with the low ionic force buffers II and III (Figure 5).

**Figure 5** - GFP expression in electroporated *S. guianensis* protoplasts in buffer I (Fromm et al., 1987), buffer II (Tada et al., 1990) and buffer III (Wlalbot, 1993). Electroporation conditions were: 500 \(\mu\)F, 100 V cm\(^{-1}\), 20 \(\Omega\) for buffer I and III, 50 \(\Omega\) for buffer II, 10 \(\mu\)g of supercoiled DNA. Bars indicate standard errors and small letters indicate significant differences at \(p < 0.05\).

**Influence of DNA form on reporter gene expression**

Figure 7 shows that linear DNA was more efficiently introduced into electroporated protoplasts. Higher
transformation efficiency using linear plasmid DNA has been reported for bacteria (Rittich and Spanova, 1996), yeasts and filamentous fungi (Kwon-Chung et al., 1998), plant protoplasts (Lin et al., 1997) and intact tissue (Akella and Lurquin, 1993; Dillen et al., 1995; Lin et al., 1997; Sabri et al., 1996; Saunders et al., 1995). Due to the inner twist of the molecule and to the absence of distortions imposed on the double helix structure (Drew et al., 1988), linear plasmid DNA is highly mobile under electric fields (Courey and Wang, 1983). Macromolecular movement across pores is facilitated when the molecules are linear because the absence of tertiary and quaternary structures reduces their volume (Tanaka, 1988) and allows a more uniform superficial polarization to be induced by the electric field (Neumann et al., 1982). The transient nature of membrane pores and of structural alterations to the cell wall induced by such electric fields imposes conformational restrictions on the type of macromolecules that can be introduced (Neumann et al., 1982).

Nandadeva et al. (1999) reported that the physical structure of DNA is critical for efficient transformation, with denatured molecules being much more favorable for transformation, although high transformation efficiency has been obtained with supercoiled plasmid DNA (Bates et al., 1990; Fromm et al., 1985; Oard et al., 1989).

In summary, we found that the critical factors regarding transgene expression in electroporated Stylosanthes guianensis protoplasts were energy input and electric field strength. A field strength of 250 V.cm⁻¹ discharged by 900 and 1000 µF capacitors gave the highest transient transformation frequencies. Linear plasmid DNA, absence of chloride and the presence of calcium ions also increased transient gene expression, although not significantly. Our data suggest that putative transgenic plants of S. guianensis can be obtained at high frequencies from electroporated protoplasts, on condition that an effective plant regeneration protocol is used.

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