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ORIGINAL PAPER

Microparticle bombardment of *Stylosanthes guianensis*: transformation parameters and expression of a methionine-rich 2S albumin gene

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Abstract An effective protocol to generate stable transformants of the tropical forage legume *Stylosanthes guianensis* (Aubl.) Sw. in a selection-free system was developed. Based on transient reporter gene expression, we have obtained transformation rates of 3.47% using 30-day-old calli as target, 1300 psi helium pressure, 12.5 cm microprojectile flight distance, 10–20 mm distance between macrocarrier membrane and stopping screen and 10–20 mm gap distance between the shock wave generator and the macrocarrier. These parameters were utilized to produce transgenic *S. guianensis* plants expressing *Be2S1* from *Bertholletia excelsa* that codes for a methionine-rich storage protein driven by a green-tissue specific promoter, *Ats1* from *Arabidopsis thaliana*. Transgenic plants were identified by a PCR-based high-throughput screen in a selective agent-free system, employing pools of 20–50 regenerating shoots. The integration of the exogenous gene in the host genome was confirmed by Southern blot analysis of PCR-positive plants. The expression of the introduced gene was confirmed in leaf tissue of transgenic plants by Northern and Western blot analyses. Immunoblots of cellular fractions showed that BE2S1 expressed in *Stylosanthes* is mainly targeted to the vacuoles.

Keywords 2S albumin · Biolistics · Genetic transformation · Legume forage · Stylo

Abbreviations

AMV	Alfalfa mosaic virus
BAP	6-Benzylaminopurine
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
CAMV	Cauliflower mosaic virus
ELISA	Enzyme-linked immunosorbent assay
GUS	β-Glucuronidase
NBT	Nitro blue tetrazolium
NEO	Neomycin phosphotransferase
NOS	Nopaline synthase
Rubisco	Ribulose 1,5-bisphosphate carboxylase/oxygenase
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis

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X-gluc	5-Bromo-4-chloro-3-indolyl glucuronide

Introduction

Stylosanthes (Fabaceae) is a large genus comprising approximately 50 species and subspecies. Most of the species, such as *S. guianensis*, *S. capitata*, *S. humilis*, *S. scabra*, *S. hamata* and *S. erecta*, are herbaceous, show vigorous growth habit, adaptability to a wide range of soil conditions and tolerance to environmental and nutritional stresses (Meijer and Szabados, 1991; Vanderstappen et al. 2003). For those reasons, these species have provided several pasture cultivars that are useful over a wide range of tropical conditions. Commercial cultivars of *S. guianensis* are employed as protein source in cattle feeding, organic fertilizer and mainly as a forage crop in consortium with a wide range of grasses in tropical regions (Kolver 2003; Rueda et al. 2003). However, low protein quality due to small amounts of essential sulfur amino acids impairs widespread utilization of *Stylosanthes* in intensive cattle feeding.

The overall protein quality in forage crops is contributed by complex metabolic pathway networks and environmental factors; thus, making it a difficult trait to be modified by traditional plant breeding. Genetic engineering provides the tools to introduce single components in more complex pathways and to regulate their expression spatial and temporally. A successful strategy to increase the nutritional value of grain crops is to introduce heterologous proteins of more adequate amino acid balance (Hagan et al. 2003; Lee et al. 2003). Low contents of methionine and cysteine are common to Fabaceae species and previous reports have shown an increase in legume protein quality by introduction of the 2S albumin gene from Brazil nut (*Bertholletia excelsa*) (Nordelee et al. 1996; Demidov et al. 2003), which has high levels of sulfur amino acids similarly to other members of the prolamin superfamily of albumin/amylase–protease inhibitor proteins (Youle and Huang 1981).

To our knowledge, *Stylosanthes* genetic transformation has only been assessed by *Agrobacterium tumefaciens* and *A. rhizogenes* (Manners 1987, 1988; Manners and Way 1989) and stable transformants have only been obtained in two reports (Sarria et al. 1994; Kelemu et al. 2005). Previous studies have shown that low transformation efficiency by *Agrobacterium*-mediated gene transfer is due to legume-bacteria incompatibility (Ankenbauer and Nester 1993; Tzifira and Citovsky 2003) and selective marker negative effect on explant shoot regeneration (Chabaud et al. 1998; Hoffmann and Vieira 2000).

We have reported direct gene transfer to *Stylosanthes guianensis* plants employing intact tissue and protoplast electroporation (Quecini and Vieira 2001; Quecini et al. 2002). Although highly efficient, both methods are labor intensive and time consuming, requiring long term in vitro cultivation before transgenic plants are obtained. Microparticle bombardment is considered the most universal genetic transformation method due to its relative independence of biological target. It is considered applicable to genetically modify a wide range of explants with high regeneration ability; reducing time, labor and costs involved in the production of transgenic plants. However, the transformation parameters must be optimized to each biological target employed (Sanford et al. 1993).

We tested the interaction between multiple physical parameters and biological target explants in order to maximize genetic transformation efficiency of *S. guianensis* by microprojectile bombardment, using a selection-free regeneration protocol. The conditions providing the highest transient transformation rates were subsequently employed to deliver a synthetic construct containing the gene coding for a methionine-rich 2S albumin under the control of a leaf-specific promoter. The present study describes the optimization of biolistic transformation of *S. guianensis* and expression analysis of 2S-expressing transgenic plants.

Materials and methods

Plant material

Scarified seeds of *Stylosanthes guianensis* cv. Mineirão were surface sterilized in 75% (v/v) ethanol for 1.5 min followed by immersion in 2% (v/v) NaOCl solution for 15 min and rinsed thoroughly four times with abundant autoclaved water. Five to 10 seeds were in vitro germinated in half-strength MS medium salts and vitamins (Murashige and Skoog 1962) plus 1.5% sucrose and 0.18% Phytagel (Sigma). Hypocotyls (~15 mm long) were excised from one-week-old seedlings and fully developed cotyledons and leaves were removed from one- and two-week-old plantlets, respectively. Leaf- and cotyledon-derived explants were also used for callus induction on MS salts medium supplemented with 2 mg l^{-1} BAP abaxial face up (Fig. 1a, b). Cultures were maintained under 16 h light regime (65 ± 3.0 μE m^{-2} s^{-1}) at 25 ± 2.5°C.

Hypocotyls, cotyledons, mature leaves and 10, 20, 30 and 45-day-old organogenic calli were assayed as microprojectile targets. Each bombardment experiment consisted of 12 plates containing 12 ml of MS medium plus 2 mg l^{-1} BAP solidified with 0.9% (w/v) Phytagel. Six explants were positioned on the bombardment plates (9 cm diameter) in order to maximize particle delivery and avoid severe tissue injury (Fig. 1c). Transformation efficiency was assessed by transient GUS expression. Each plate was considered a replicate and the frequency of GUS positive explants was evaluated in three categories: less than five GUS-positive foci per explant, between five and 50 GUS-positive foci per explant and more than 50 GUS-positive foci per explant.

Immediately after bombardment, the explants were transferred to hormone-free MS salts and vitamin medium containing 3% (w/v) sucrose and 0.18% (w/v) Phytagel. After approximately three weeks, 2–4-cm elongated shoots (Fig. 1d) were individualized by basal excision and transferred to rooting medium, consisting of hormone-free MS salts and vitamins solidified with 0.18% (w/v) Phytagel and added with 1% (w/v) of sucrose. Typically, rooted individualized plants were obtained 55 days after the bombardment.

Leaf segments from rooted plantlets (Fig. 1e) were removed and employed for histochemical GUS and PCR analyses. Plants expressing the *GUS* gene and positive in the PCR analysis were transferred to plastic pots containing autoclaved vermiculite for 3–4 weeks. Further acclimatization was carried out successively to plastic and ceramic pots, both containing a mixture of soil: sand: manure (1:1:1) during 2–3 week time.

Plasmids

In order to optimize physical and biological parameters of the transformation pBI426 (provided by Dr. W. Crosby, Plant Biotechnology Institute, Saskatoon, Canada) was employed. Its expression cassette carries the transcriptional fusion of the *GUS* and the *NEO*, conferring kanamycin resistance, genes driven by a double 35S promoter from CaMV plus the 5′ untranslated sequence of AMV, which acts as a transcriptional

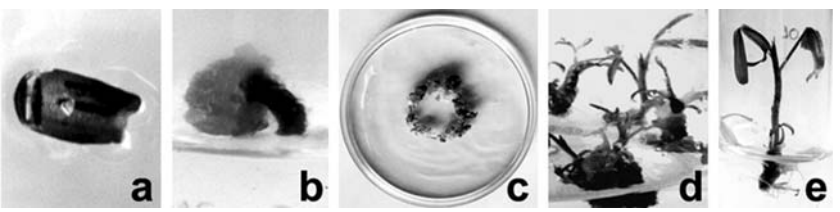

Fig. 1 Organogenic regeneration of *Stylosanthes guianensis* calli in microprojectile bombardment transformation system. (**a**) cotyledon-derived explant on callus-inducing medium, (**b**) 20-day-old callus, (**c**) organogenic calli positioned on bombardment plate, (**d**) shoot regeneration from bombarded calli and (**e**) isolated shoot in root-inducing medium

enhancer, and the NOS terminator from *Agrobacterium tumefaciens*.

The vector pEAats:2S contains the coding sequence of the methionine-rich 2S albumin—*Be2S1* (Gander et al. 1991) from Brazil nut (*Bertholletia excelsa*) driven by the rubisco small subunit promoter, *ats1A* from *Arabidopsis thaliana,* promoting transcription in a chlorophyll-biosynthesis dependent manner (Almeida et al. 1989). This cassette also contains a translational fusion of the genes *GUS* and *NEO* driven by *NOS* promoter and terminator. Plasmids were transformed into *E. coli* JM 109 cells and purified by gradient ultracentrifugation (Sambrook et al. 1989). Supercoiled plasmid DNA was directly employed in transformation experiments. Concentrations of plasmid DNA were determined spectrophotometrically at 260 nm and confirmed by electrophoresis through 0.8% agarose gels stained with ethidium bromide viewed under ultraviolet light (Sambrook et al. 1989).

Biolistic transformation

A commercial high-pressure helium-driven microprojectile acceleration device PDS1000/He™ (Bio Rad) was utilized (Kikkert 1993). Tungsten 1.2 μm-diameter microparticles (M10, Sylvannia Inc.) were coated with 5 μl of plasmid DNA (1 μg μl^{-1}) using 1 M $CaCl_2$ and 0.1 M spermidine as described (Aragão et al. 1996) under low relative humidity conditions (less than 40%). Vacuum inside the acceleration chamber was maintained at 68 mmHg.

Regeneration-competent explant sources, namely: hypocotyls, cotyledons, leaves and 10-, 20-, 30- and 45-day-old calli, were assayed and the biological targets providing higher transient reporter gene expression were employed for testing the subsequent parameters.

Three helium pressures (1000, 1300 and 1600 psi) interacting with two-target distances (12.5 and 5 cm) were tested. The interaction between macrocarrier flight distances and helium gas pressures were tested employing four distinct pressures (1000, 1300, 1600 and 2000 psi) and two macrocarrier levels (0.1 and 0.2 cm). The gap between the gas acceleration device and the macrocarrier was also tested in interaction with the helium pressure, employing the same two gap distances and four gas pressures.

A set of three independent experiments was carried out to test biological and physical parameters involved in microprojectile plant transformation as suggested (Sanford et al. 1993). The mean of 12 bombardment plates containing six explants was used to compare the treatments. The highest levels of transient *GUS* gene expression were obtained with leaf-derived organogenic calli (30 days old), 1300 psi, 12.5 cm of target distance, 0.1 cm macrocarrier level and 0.1 cm gap distance. These conditions were employed subsequently in 12 independent trials with transformation vector pEAats:2S. In each experiment, 12 plates containing six explants were bombarded, totalizing 864 calli.

Histochemical GUS assays

Explants were analyzed for β-glucuronidase activity as described (Jefferson et al. 1987). Bombarded tissues were vacuum-infiltrated for 5 min with the staining buffer containing the chromogenic substrate, X-Gluc (McCabe et al. 1988) to which 20% (v/v) methanol was added in order to inhibit endogenous β-glucuronidases (Kosugi et al. 1987). After 24-h incubation at 37°C, tissue pigments were removed by successive washes with 80% (v/v) ethanol and the explants were observed under the stereoscope. GUS stained areas were visually evaluated and grouped into three categories according to the number of blue-stained foci: <5 foci per explant, $5<$ foci per explant <50 and >50 foci per explant.

Nucleic acids analysis

Regenerating shoots were screened by the specific amplification of a 373 bp fragment from *Be2S* in pooled PCR assays. Each 25 μl reaction mixture contained 20 ng of genomic DNA pooled from 20 to 50 regenerants (Edwards et al. 1991), 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 160 μM of each dNTP, 1.0 U of *Taq* polymerase (Invitrogen) and 200 nM of gene specific primer. The primers used to amplify the methionine-rich gene (*Be2S1*) were: 5′-CACAGTGGTGGAGGAGAA-3′ and 5′-TCGGCCAGCCTCATCATC-

CTT-3′. Mixtures were submitted to initial denaturation (5 min at 95°C) followed by 35 cycles of amplification (1 min at 94°C, 1 min at 45°C and 1.5 min at 72°C) and final extension (7 min at 72°C) in thermal cycler (Perkin Elmer Thermus). The amplification products were run in 1.4% (w/v) agarose gels.

For Southern blot hybridizations, total genomic DNA was isolated from leaf tissue of putative transgenic primary regenerants (R_0) and the controls following standard procedures (Dellaporta et al. 1983). Southern blotting was performed as described (Sambrook et al. 1989): ca. 15 μg of DNA were digested to completion with *Eco*RI (Invitrogen, Carlsbad CA, USA), electrophoresed and transferred onto Hybond N^+ membranes (GE Healthcare, Fairfield CT, USA). Similarly, total RNA was isolated from 2 g of tissue from the primary transformants and the controls, as described (Jones et al. 1985) and quantified by spectrophotometer readings at 260 nm. Ten μg of total RNA were size-resolved in denaturing formaldehyde (2.2 M) gels (MOPS 1×, 1.25% (w/v) agarose) and blotted onto Hybond N^+ membranes (GE Healthcare, Fairfield CT, USA).

A 4.3 kb fragment, corresponding to the *ats1A* promoter and the *BE2S1* coding sequence was obtained by digestion of 10 μg of the transformation vector pEAats:2S with *Eco*RI. The fragment was purified by electroelution (45 min, 10 mA per tube) using Electro Eluter 422 (Bio Rad, Hercules CA, USA). The isolated fragment was [γ-^{32}P]dATP-labeled by Prime-a-Gene Labeling System (Promega, Madison WI, USA) according to the manufacturer's instructions and used as the probe. Membranes were hybridized as describe (Memelink et al. 1994).

Protein expression analysis

Bertholletia excelsa embryos were defatted, finely ground in liquid N_2 and total proteins were extracted as described (DeClerq et al. 1990). Lower sedimentation coefficient albumins (6S–2S) were separated by ultracentrifugation in a sucrose continuous gradient (0–20% w/v) (Martin and Ames 1961) and size resolved on gradient 8–18% (v/v) SDS-PAGE. The 12 kD band, corresponding to the protein BE2S1, was excised from the gel and electroeluted at 10 mA per tube for 5 h at 4°C. The purified protein was quantified (Bradford 1976) and injected, at 1 μg per animal, in female BALB/c mice for three consecutive weeks, plus a 5 μg boost application. The serum anti-2S titer was monitored by ELISA and antibody purification was performed as described (Harlow and Lane 1988). *Stylosanthes* proteins were isolated from whole-leaf tissue and from protoplasts and their corresponding subcellular fractions (DeClerq et al. 1990). Mesophyll protoplasts were isolated as previously described (Quecini and Vieira 2001), cultivated for 48 h in K8P medium (Kao 1977) and employed for subcellular fractioning and vacuole isolation (Glund et al. 1984). The purity of the vacuolar fraction was evaluated by microscopic observation. Protoplast- and subcellular fraction-proteins were submitted to dialysis for 24 h at 4°C (DeClerq et al. 1990) to remove the osmoticum. For immunoblots, 30 μg of total protein were run on 8–18% (v/v) gradient SDS-PAGE and transferred onto nitrocellulose membranes (Hybond C, GE Healthcare, Fairfield CT, USA) using a semi-dry transfer apparatus (TransBlot, Bio Rad) at 10 V for 60 min. The proteins were visualized by incubating the membrane treated with primary polyclonal antibody against BE2S1 with anti-mouse alkaline phosphatase-conjugated secondary antibody (Promega) in the presence of BCIP and NBT as substrates.

Results and discussion

Biological parameters

In order to optimize the efficiency of microprojectile gene transfer to *S. guianensis*, we have tested the following explants: hypocotyls, cotyledons, leaves and 10-, 20-, 30- and 45-day-old calli. All the explant types of *S. guianensis* used as biological targets have high organogenic regenerability in vitro (Fig. 1), using the protocol previously described for *S. scabra* (Dornelas et al. 1992). The results of three independent experiments, consisting of 12 plates with six explants each, were evaluated for transient expression of a reporter gene. Each plate was considered a

replicate and the frequency of GUS positive explants was classified in three categories: less than five, between five and 50 and more than 50 GUS-positive foci per explant.

For hypocotyls and leaves the frequency of explants expressing the *GUS* gene was low (more than 50% of the bombarded explants showed no GUS staining) in all three experiments (Fig. 2a, b). Most of the hypocotyls- and leaf-based explants showed less than five foci of GUS expression (Table 1) and the intensity of GUS-staining was weaker in hypocotyls compared to the intensity in transiently transformed leaves (Fig. 2b). Intermediate levels of *GUS* gene expression and frequencies of transiently transformed explants were observed in cotyledon-derived explants and 10-, 20-day-old calli (Fig. 2b, Table 1). However, the expression was diffuse, suggesting that the product of the staining reaction was transported from transformed cells to the neighboring ones. In contrast, practically all 30- and 45-day-old calli showed more than 50 GUS-stained spots (Table 1). The expression level, evaluated as the intensity of the GUS staining, was also higher in calli when compared to the level in the other explants tested (Fig. 2d).

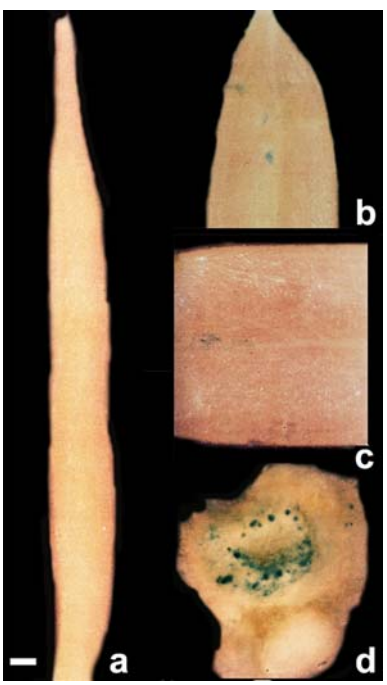

Fig. 2 Transient GUS expression in different bombarded *Stylosanthes guianensis* explant types: (**a**) hypocotyl, (**b**) cotyledon, (**c**) leaf, (d) 30-day-old callus. The bar represents 0.25 cm

The biological target was critical for transformation efficiency. Explants with restrict surface gave almost null levels of transformation. In leaves and stems the reduced surface and thickness of the explant caused the frequency of transient expression of the reporter gene to be low. On the other hand, organogenic calli had nearly 100% of reporter gene expression. This could be due to a larger exposed surface as target and the friable nature of the explant, favoring the penetration of the microprojectiles. The efficiency of stable transformation by microprojectile bombardment depends on the ability of the target explant to regenerate whole plants (Kohli et al. 2003). The high regenerability of *Stylosanthes* organogenic calli has previously been shown (Dornelas et al. 1992) and was confirmed by this study with *S. guianensis* (Fig. 1). Thus, 30-day-old calli were employed as microparticle target explant in the optimization of the physical parameters of transformation and in the actual stable transformation experiments due to its high in vitro regenerability and reporter gene expression in transient gene transfer experiments.

Physical parameters

The physical parameters of biolistic transformation were evaluated as pair-wise combinations so that the interaction between them could be assessed. Three helium (He) pressures (1000, 1300 and 1600 psi) combined with two microparticle-flight distances (12.5 and 5.0 cm) were tested, with macrocarrier flight distance and gap between shock wave generator and macrocarrier of 0.1 cm. The average results of three independent experiments, consisting of 12 plates with six explants each, were used to evaluate the frequency of transient expression of the reporter gene.

The highest frequency of GUS-expressing explants was obtained combining 1300 psi of He with a flight distance of 12.5 cm (Table 2). Approximately 95% of the bombarded calli showed more than 50 GUS-stained foci. A He pressure of 1600 psi caused intense tissue damage regardless of the flight distance used and thus, the reporter expression level was low (Table 2).

Table 1 Transient GUS expression in *Stylosanthes guianensis* according to the explant-type

Explant Type	GUS positive explants (% ± se)		
	Class I <5 foci per explant	Class II 5< foci per explant <50	Class III >50 foci per explant
Leaf	85.23^{c} ± 1.02	2.45^{a} ± 0.04	12.32^{b} ± 0.15
Hypocotyls	98.65^{c} ± 1.12	1.35^{a} ± 0.10	0
Cotyledons	8.69^{a} ± 0.42	6.11^{a} ± 0.94	85.20^{a} ± 1.01
10-Day-old calli	3.20^{a} ± 0.27	3.46^{a} ± 0.29	93.34^{c} ± 0.75
20-Day-old calli	5.14^{a} ± 0.61	2.79^{a} ± 0.32	92.07^{c} ± 0.95
30-Day-old calli	0	0	100^{c} ± 0.12
45-Day-old calli	0	0	100^{c} ± 0.20

Percentage values are the average of three independent bombardment experiments ± standard error (se). The statistical significance of the treatments at 1% of probability (non-parametric Tukey test) is represented by small caps letters

Table 2 Physical parameters affecting microprojectile-mediated gene transfer to *Stylosanthes guianensis*

Physical parameter	GUS positive explants (% ± se)		
	Class I <5 foci per explant	Class II 5< foci per explant <50	class III >50 foci per explant
He pressure (psi)—target distance (cm)			
1000-12.5	0	76.34^{f} ± 1.85	23.66^{b} ± 0.99
1000-5.0	0	67.57^{e} ± 0.68	32.43^{c} ± 0.54
1300-12.5	0	5.56^{a} ± 1.03	94.44^{f} ± 0.95
1300-5.0	0	85.13^{f} ± 0.87	14.87^{b} ± 1.10
1600-12.5	8.19^{a} ± 0.65	12.03^{a} ± 1.09	79.78^{f} ± 0.79
1600-5.0	18.02^{b} ± 1.21	58.11^{d} ± 1.46	23.87^{b} ± 1.88
He pressure (psi)—macrocarrier flight distance (cm)			
1000-0.1	0	0	100^{e} ± 0.64
1000-0.2	0	0	100^{e} ± 0.19
1300-0.1	0	0	100^{e} ± 0.45
1300-0.2	0	0	100^{e} ± 0.33
1600-0.1	0	0	100^{e} ± 1.76
1600-0.2	3.97^{a} ± 0.21	4.66^{a} ± 0.47	91.37^{e} ± 1.11
2000-0.1	16.17^{b} ± 0.87	83.83^{e} ± 1.21	0
2000-0.2	38.55^{c} ± 0.69	61.45^{d} ± 0.17	0
He pressure (psi)—gap between shock wave generator and macrocarrier (cm)			
1000-0.1	0	0	100^{e} ± 0.45
1000-0.2	0	0	100^{e} ± 0.37
1300-0.1	0	0	100^{e} ± 0.67
1300-0.2	0	0	100^{e} ± 0.95
1600-0.1	0	0	100^{e} ± 1.05
1600-0.2	4.02^{a} ± 0.13	3.67^{a} ± 0.09	92.31^{e} ± 0.79
2000-0.1	18.55^{b} ± 0.34	81.45^{e} ± 1.05	0
2000-0.2	34.15^{c} ± 0.67	65.85^{d} ± 0.78	0

Percentage values are the average of three independent bombardment experiments ± standard error (se). The statistical significance of the treatments at 1% of probability (non-parametric Tukey test) is represented by small caps letters

Intermediate expression levels were obtained with lower pressure, however the dispersion of the microprojectiles was restricted and explants with more than 50 foci of staining were infrequent (Table 2).

The acceleration of the particles towards the target is influenced by the interaction between the pressure of He and the flight distance of the macrocarrier (Sanford et al. 1993). In the present study, we have tested the following combinations of He pressure (psi) and macrocarrier membrane flight distance (cm): 1000 and 0.1; 1000 and 0.2; 1300 and 0.1; 1300 and 0.2; 1600 and 0.1; 1600 and 0.2; 2000 and 0.1; 2000 and 0.2, in three

independent experiments consisting of 12 plates with six explants while keeping the target at 12.5 cm. The average frequency of transiently transformed explants was high for all the tested combinations, except for the treatments with He pressure of 2000 psi (Table 2). There were no significant differences among the treatments using helium pressures of 1000, 1300 and 1600 psi, where approximately 97% of the explants had more than 50 foci of GUS expression (Table 2).

The dispersion and the acceleration of the particles can be affected by the interaction between the flight distance of the macrocarrier membrane and the distance between the chamber where the shock wave is generated and the rupture screen (Sanford et al. 1993). Smaller distances increase the acceleration but also cause more variation in the microparticle trajectory; thus, increasing the particle delivery to suboptimal regeneration regions in the target explant. We have tested pair-wise combinations of the previously employed He pressures and 0.1 or 0.2 cm of distance between the shock wave chamber and the macrocarrier membrane in three independent transformation experiments, consisting of 12 plates with six calli each with a microprojectile flying distance of 12.5 cm. He pressure had the stronger effect on the number of GUS-expressing explants, since the frequency of calli showing GUS staining was markedly smaller at 1600 and 2000 psi of pressure, regardless of the distance between the shock wave generator and the macrocarrier (Table 2). High pressure-induced damage to the explant has also been detected (Table 2). Thus, the macrocarrier flight distance and the gap between shock wave generator and macrocarrier did not have critical influence on the transformation efficiency.

The physical parameters leading to the highest frequency of GUS-stained explants consisted of 1300 psi of He pressure, microparticle flight distance of 12.5 cm, macrocarrier membrane flight distance of 0.1 cm and distance between the shock wave generator and the macrocarrier of 0.1 cm. These parameters were further utilized for the stable transformation experiments.

Stable transformation

The biological and physical parameters that provided the highest levels of transient transformation were employed in 12 subsequent experiments using the transformation vector pEAats:2S that carries the coding sequence of 2S albumin from *Bertholletia excelsa* driven by a green tissue-specific promoter. The vector pEAats:2S carries a fusion between a selective marker gene, *NEO*, conferring kanamycin resistance and the reporter gene *GUS*, however, regeneration was carried out in the absence of the selective agent kanamycin in this study. Instead, we have devised a PCR-based high-throughput screen where the DNAs from 20 to 50 regenerating plants were pooled and analyzed for the transgene amplification. The regenerated plants were individually analyzed in case of a positive result for the amplification of the *Be2S1*-specific fragment in the pool PCR reaction. Typically, shoot regeneration initiated nine days after bombardment and continued for approximately 45 days. Each explant generated an average of 3.46 shoots along this period (Table 3).

Thirty stable transformants were obtained from 864-bombarded calli, resulting in a transformation efficiency of 3.47% (Table 3). The transformation efficiency of Fabaceae by microparticle bombardment has been reported to be of approximately 1% (Atkins and Smith 1997; Somers et al. 2003). In selection-free regeneration systems, it is more difficult to identify stably transformed plants due to the dilution effect caused by the growth of non-transformed regenerants (Klaus et al. 2003; Sato et al. 2004). Thus, if transformation efficiency is calculated based on the number of regenerants analyzed (2990) (Table 3), it drops to approximately 1%, which remains close to the average transformation efficiency in non-model legume system (Atkins and Smith 1997; Somers et al. 2003). The main advantage of the selection-free regeneration is the absence of resistance genes (e.g. to antibiotics or herbicides) in the final product, reducing concerns about horizontal gene transfer of resistance genes to plant-borne microorganisms (Schlüter et al. 1995; Bennet et al. 2004) and to wild species (Fontes 2003) or about increasing selective

Table 3 Stable transformation of *Stylosanthes guianensis* by microparticle bombardment in the absence of selective agent

Transformation experiment	Explants	Regenerants	Regenerants/explant (average ± se)	Transgenic plants
1	72	249	3.43	2
2	66	231	3.50	0
3	72	285	3.65	3
4	78	185	2.57	0
5	72	247	3.46	3
6	72	264	3.67	3
7	72	251	3.48	5
8	78	272	3.49	3
9	78	284	3.64	5
10	60	227	3.78	1
11	72	248	3.44	3
12	72	247	3.43	2
Total	864	2990	3.46 ± 0.09	30

Microparticle bombardment conditions were: 1000 psi He, 65 mmHg vacuum, 10 mm gaps, and 12.5 cm target distance. The average number of regenerants per explant is presented as mean ± standard error (se)

pressure (Schulz et al. 1990; Bennet et al. 2004; Fontes 2003; Mascia and Flavell 2004).

The primary transformants (R_0) showed *GUS* gene expression in all analyzed tissues and the presence of the 4.3 kb band corresponding to the plant transcription unit integrated to the genome (Fig. 3).

Number and structure of the inserts

The copy number and the complexity of the transgene integration in the host genome was analyzed by probing *Eco*RI digested genomic DNA from the putative transformants with a 4.3 kb fragment from the transformation vector, corresponding to the regulatory untranslated regions and the coding sequence of *Be2S1*. The transgene was detected in the genome of all GUS-expressing regenerants (Fig. 3). Wild-type control plants did not show signal of hybridization with the plant transcription unit employed as probe.

The ratio between the size of *S. guianensis* haploid genome (Arumuganathan and Earle 1991) and the plant transcription unit from pEAats:2S was used to determine the stoichiometric amount of the plasmid DNA generating a hybridization signal equivalent to one copy of the transgene inserted. Thus, this amount of transformation vector was used a copy number control. Comparing the signal intensity, most of the transformants (90%) presented more than 1 copy of the inserted gene (Fig. 3). The insertion of

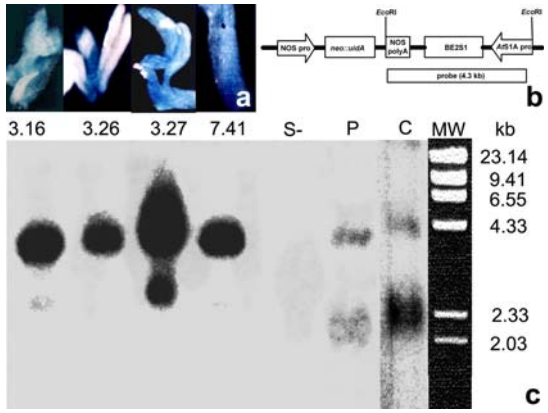

Fig. 3 Southern blot and GUS expression analyses of the *Stylosanthes guianensis* plants transformed with pEA*ats*:2S by bombardment. (**a**) GUS expression in primary transformant (T0) leaf segments. (**b**) schematic representation of the plant transcription unit from pEAats:2S and hybridization probe (pro: promoter, polyA: polyadenylation signal). (**c**) Southern blot analysis of T0 plants. The numbers above the lanes are the plant designation, S-: non-transformed *S. guianensis* DNA, P: pEAats:2S plasmid DNA in an amount corresponding to 1 copy of transgene per haploid genome of *S. guianensis*, C: *Bertholletia excelsa* genomic DNA (15 μg), MW: molecular weight marker (λ*Hin*dIII, GIBCO-BRL)

multiple copies of the transgene in the host genome has been frequently associated to direct transformation methods, due to the non-specific nature of the physical forces driving the insertion and the large amount of plasmid DNA employed (Christou et al. 1989; Matzke and Matzke 1995).

The presence of smaller bands (approximately 2.0 kb and 500 bp) is not likely to be resultant

from cross-hybridization of the probe with endogenous stylo genes coding for small molecular weight storage albumins that have been to shown to belong to highly conserved gene families among different species (Guerche et al. 1987; Krebbers et al. 1988), due to its absence from the non-transformed control. Alternatively, it is likely to have resulted from the partial integration of the transformation vector or fragmentation of the plasmid DNA during the transformation process (De Block 1993).

Expression of the transgene

The primary transformed plants of *Stylosanthes* showed expression of the transgene in leaf-tissue Northern blots (Fig. 4). No signal was observed when RNA isolated from stems or roots was probed (data not shown). The observed pattern is in accordance with the light-regulated, chlorophyll biosynthesis-dependent promoter used in the chimerical construct. Similarly, 2S albumin (12 kD) was detected in the leaves of the transgenic plants by Western blot assays using a polyclonal antibody against Brazil nut 2S (Fig. 5). Immunologically related polypeptides of smaller sizes were absent from the transgenic *S. guianensis* plants suggesting that the proteolytic processing of 2S albumins remains unaltered. Similar translational expression profiles of BE2S1 were observed in transgenic *Vicia narbonensis* (Saalbach et al. 1994, 1995).

Storage proteins are transported to protein bodies, subcellular specialized compartments

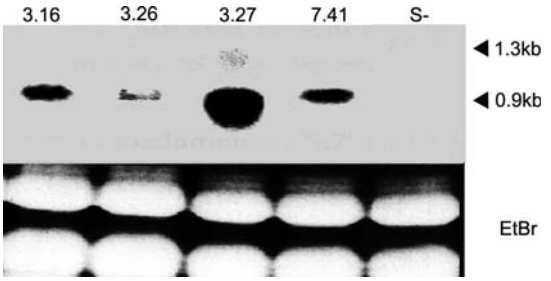

Fig. 4 Northern blot analysis of the *Stylosanthes guianensis* plants transformed with pEA*ats*:2S by bombardment. The numbers above the lanes indicate the transformed plants, S-: non-transformed *S. guianensis* RNA, Etbr: UV-picture of etidium bromide-stained gel as loading control

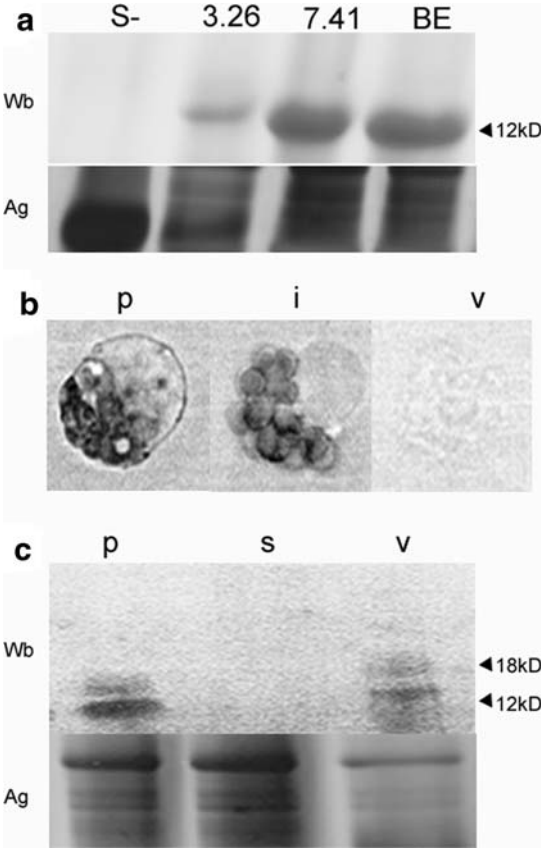

Fig. 5 BE2S1 expression in transgenic *Stylosanthes guianensis* plants. (**a**) 2S albumin expression in *S. guianensis* leaves. The numbers above the lanes indicate the transformed plants, S-: non-transformed *S. guianensis*, BE: total soluble proteins from *Bertholletia excelsa* embryos. Wb: western blot, Ag: silver staining of a similar gel as loading control. (**b**) subcellular fractioning; p: isolated protoplast from *S. guianensis* leaves, i: vacuole release upon cellular disruption and v: isolated vacuole. (**c**) Immunological analysis of BE2S1 in the subcellular fractions of plant 3.26 using an anti-2S polyclonal antibody. Wb: western blot, Ag: silver staining of a similar gel as loading control

similar to vacuoles exclusively found in seed, via the secretory pathway (Vitale and Hinz 2005). This process is submitted to several steps of protein quality control and incompletely or miss-processed proteins are targeted to degradation (Huber and Hardin 2004). In order to identify the subcellular localization of the heterologous protein in *Stylosanthes* leaves and to determine its integrity in the final storage compartment, we performed immunological assays of cellular fractions of the transgenic plant leaves. The characteristic 18 kD BE2S1 propeptide was identified in

intact protoplasts and in the vacuolar fraction (Fig. 5). The propeptide has been shown to contain the vacuolar targeting signal (Saalbach et al. 1996), in agreement with the detection of approximately 67% of the heterologous BE2S1 in the vacuolar fraction of *Stylosanthes* leaves (Fig. 5).

In the present work we have shown the stable integration and expression of an exogenous transgene in the genome of *S. guianensis* plants by microparticle bombardment. The transformation efficiency of 3.74% is higher than the reported average for direct transformation methods in non-model legume crops and selection-free transformation has been shown to be feasible. Transgene expression was detected in the leaves and the exogenous seed storage protein is preferentially targeted to vacuoles. Taken together, these results demonstrated that genetic engineering can increase the protein quality in rustic forage legume crops.

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