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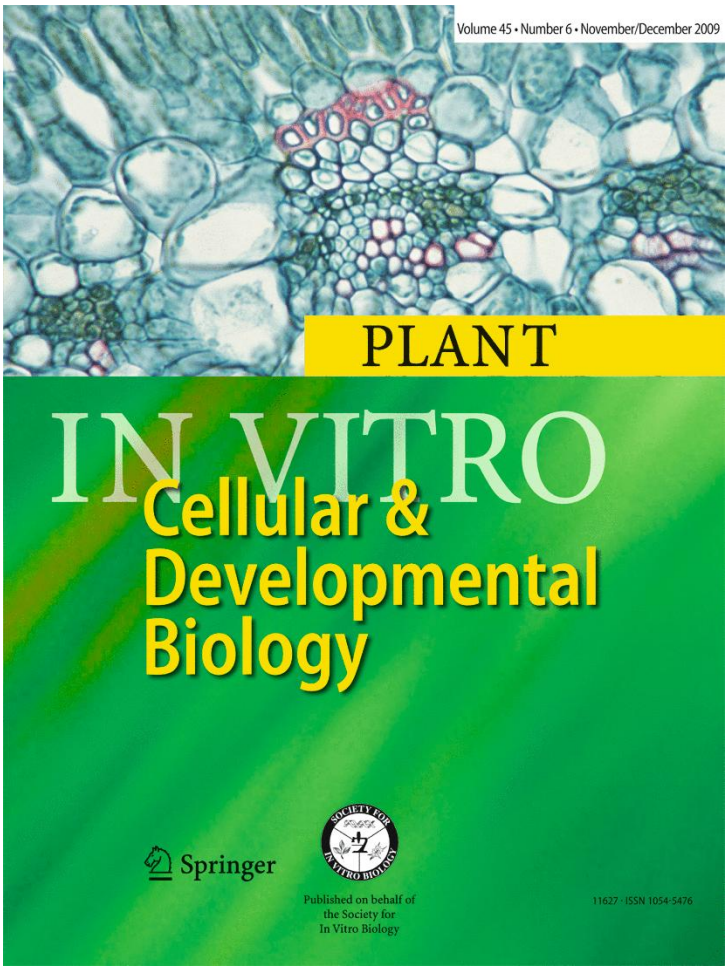

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GENETIC TRANSFORMATION

Genetic transformation of blue grama grass with the *rolA* gene from *Agrobacterium rhizogenes*: regeneration of transgenic plants involves a "hairy embryo" stage

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Abstract Until recently, information about the effects of transforming plants with the *rolA* gene of *Agrobacterium rhizogenes* has been restricted mainly to dicots in which a severely wrinkled phenotype, reduced internode distances, and abnormal reproductive development were commonly observed. In this work, we analyzed the effects associated with the expression of this gene in a new genetic context: the forage grass genome. Transgenic P_{35S}•*rolA* plants of blue grama grass (*Bouteloua gracilis*) were obtained by a biolistic approach employing embryogenic chlorophyllic cells as the target material. Four independent transgenic lines with regeneration capacity were recovered, which showed stable integration of this transgene as demonstrated by polymerase chain reaction and Southern blot hybridization. Growth of the *rolA*-transformed lines under greenhouse conditions provided evidence for a new biotechnological application for the *rolA* gene in plants, namely, the improvement of biomass production in forage grasses. Additionally, we described here a new phenotypic marker (referred to here as the "hairy embryo" syndrome) that can be instrumental for the early identification of transformation events when transforming grasses with this gene.

Keywords *rolA* · *Agrobacterium rhizogenes* · *Bouteloua gracilis* · Forage grass · Cereals · Tillering · Genetic transformation · Biolistics

Introduction

Agrobacterium rhizogenes is a gram-negative, soil-borne bacterium capable of inducing root formation in infected tissues (Elliot 1951). This process is mediated by the transfer of a DNA segment found in pRi (root-inducing)

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plasmids known as transfer DNA (T-DNA), which, in agropine-type *A. rhizogenes* strains, is divided into two subfragments named leftward transfer DNA (TL-DNA) and rightward transfer DNA (TR-DNA), each coding for specific morphogenetic activities.

Dicotyledonous transgenic plants containing the complete Ri TL-DNA often display a condition referred to as the "hairy root" syndrome or Ri phenotype, which is characterized by wrinkled leaves, shortened internodes, reduced apical dominance, increased branching, plagiotropic root systems, heterostyly, partial sterility, and conversion from biennials to annuals (Christey 2001; Casanova et al. 2005; Bulgakov 2008). It has been demonstrated that most of these phenotype modifications are the result of the expression of specific genes from the leftward subfragment known collectively as *rol* genes (Durand-Tardif et al. 1985; Ooms et al. 1986; Vilaine et al. 1987; Schmülling et al. 1988; Cardarelli et al. 1989). The genetic content of TL-DNA includes approximately 18 potential genes (Slightom et al. 1986), of which only the *rol* genes, open reading frames 10 (*rolA*), 11 (*rolB*), 12 (*rolC*), and 15 (*rolD*), have been implicated in the establishment of the "hairy root" syndrome and studied to a certain extent (White et al. 1985; Spena et al. 1987).

Although this syndrome is caused by their synergistic action, the *rol* genes exert particular effects on plant phenotype, which are not only species-dependent (Van der Salm et al. 1996) but also clone-dependent and individual-dependent (Tepfer 1984). Consequently, within a species context, the effects of these genes are strongly linked to the structure, growth habit, developmental pattern, and other intrinsic characteristics of the transformed organism. Because every *rol* gene is responsible for specific alterations of the "hairy root" syndrome, these genes are considered to be powerful tools for altering plant morphology and functioning with practical or scientific purposes (Christey 2001; Casanova et al. 2005). In particular, transformation of dicots with the *rolA* gene results in decreased plant stature (shortened internode length), green darkened leaves, highly ramified stems (reduced apical dominance), leaf wrinkling, decreased length-to-width leaf ratio, hypostyle (shortened styles), larger flower size, reduced flower number, condensed inflorescences, male sterility, and retarded onset of flowering (Schmülling et al. 1988; Sinkar et al. 1988a; McInnes et al. 1991; Sun et al. 1991; Van Altvorst et al. 1992; Carneiro and Vilaine 1993; Dehio et al. 1993). These effects (except male sterility) are enhanced in plants transformed by the *rolA* gene under the control of the 35S promoter (Dehio et al. 1993). Although the biochemical function of *rolA* is unknown (Bulgakov 2008), at least some of the alterations produced by the pleiotropic action of the *rolA* gene seem to be the result of changes in hormone physiology (Maurel et al. 1991; Sun et al. 1991; Vansuyt et al. 1992; Dehio et al. 1993; Schmülling et al. 1993; Prinsen et al. 1994).

Most information concerning the effects of *rol* genes on the structure and functioning of plants has been mainly restricted to dicots because of the well-known natural incapacity of *Agrobacterium* for transforming monocots, particularly graminaceous plants (cereals and grasses). More recently, Lee et al. (2001) successfully transformed rice with a construct containing *rolA* under the control of a double 35S promoter (P_{35S}•*rolA*) via polyethylene glycol (PEG)-mediated protoplast transformation. Some alterations detected in these transgenic plants of rice, such as dark green wrinkled leaves, resembled those reported previously for dicots. This pioneer work opened the possibility of understanding the effects of *rol* gene expression on the structure and development of plants in the Poaceae.

In the present research, we transformed a very important forage grass, native to the North American Desert, *Bouteloua gracilis* (H.B.K.) Lag. ex Steud. with the *rolA* gene under the control of the 35S promoter in order to determine the effects associated to the expression of this gene in a forage grass genetic context. P_{35S}•*rolA* transgenic plants were obtained by a biolistic approach using a well-developed protocol for transforming embryogenic chlorophyllic cells. New insights are provided on the effects of the *rolA* expression in the *B. gracilis* genome. *rolA* transgenic lines displayed a "hairy embryo" syndrome, characterized by the development of very conspicuous pubescence at the proximal region of regenerating transgenic embryos. Plants maintained this hairiness until the adult phase and were sterile. More important, the transformed lines showed a reduced root system, but they produced up to twice as much foliage as control plants. We discuss the relative impacts of the traits associated with *rolA* expression in monocot and dicot genomes.

Materials and Methods

"TADH-XO" cell line establishment and maintenance. The embryogenic, highly chlorophyllous "TADH-XO" cell line (García-Valenzuela et al. 2005) was obtained from culturing shoot apices-derived green calluses in liquid propagation medium containing the salts of the MS medium (Murashige and Skoog 1962) plus 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid, 2 mg L^{-1} N^6-benzylaminopurine, and 40 mg L^{-1} adenine, as described previously (Aguado-Santacruz et al. 2001). This cell line was subcultured every 20 d by transferring 1 ml of the cell suspension into 24 ml of fresh propagation medium. All reagents employed in this study were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Plasmid DNA. We transformed the chlorophyllous embryogenic cells of blue grama with plasmid pLBR29 (Fig. 1), provided by Dr. David Tepfer of INRA, France. This plasmid was previously used by Lee et al. (2001) for transforming rice and contains the *rolA* sequence from the TL-DNA of *A. rhizogenes*, under the control of a double cauliflower mosaic virus (CaMV) 35S promoter (P_{35S}) and the CaMV 35S terminator ligated into the *Kpn*I/*Xba*I sites of the cloning vector pRT99, which carries the *nptII* gene as a selectable marker (Töpfer et al. 1988).

Microprojectile bombardment of blue grama embryogenic cells. Chlorophyllous cells from a 6-d-old suspension culture were distributed onto 3.0 cm diameter paper filter disks (approximately 2 g FW cells). One day prior to bombardments, cells were osmotically stressed by transferring the cells onto propagation medium containing 0.4 M mannitol. This osmotic stress treatment has been shown to increase the transformation efficiency of the chlorophyllic cells (Aguado-Santacruz et al. 2001). The filter disks containing the stressed cells were centered onto the Petri dishes and prepared for bombardments by either the particle inflow gun (PIG; Finer et al. 1992) or the biolistic particle delivery system (PDS-1000; Sanford et al. 1991). The initial preparation of bombardment mixtures used for both biolistic systems was as follows: 50 µl M10 tungsten particles (15 mg mL^{-1}), 10 µl DNA (1 µg mL^{-1}), 50 µl 2.5 M $CaCl_2$, and 20 µl 0.1 M spermidine were mixed in sequential order. The entire mixture was then vortexed for 5 min and briefly sonicated. This mixture was subsequently centrifuged at 10,000×*g* for 10 s. After this latter step,

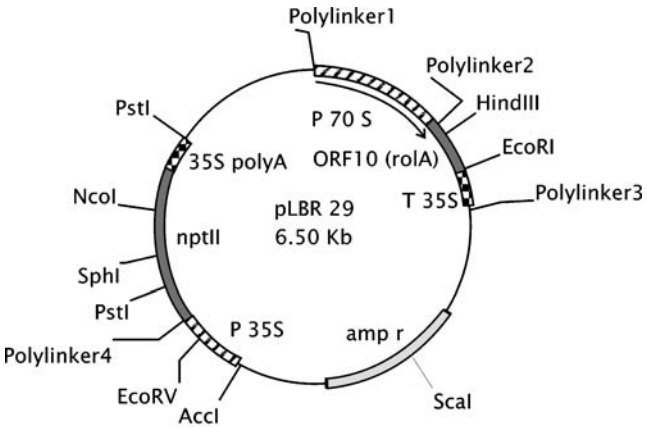

Figure 1. Diagram of the pLBR29 plasmid containing the *rolA* gene under the control of a double 35S CaMV promoter. The selective marker *nptII* is flanked by sequences of the promoter and terminator of the CaMV.

bombardment DNA preparations were treated differently for the PIG and PDS-1000 systems. For PIG, 60 µl of the supernatant were discarded and the rest dispensed into 5 µl aliquots for individual shots. The particle/DNA mixture was placed in the center of the syringe filter unit. The embryogenic cells were covered with a 500-µm baffle, placed at a distance of 10 cm from the screen filter unit containing the particles and bombarded once with a pressure of 80 psi. The vacuum chamber was maintained at 60 mmHg.

For PDS-1000, 60 µl of absolute ethanol were added to the DNA–tungsten particle mixture after the above-mentioned centrifugation step. This mixture was then sonicated and centrifuged for another 10 s at 10,000×*g*. The supernatant was discarded and the pellet was again resuspended by sonication in 75 µl absolute ethanol; 10-µl aliquots of this final mixture were loaded onto each macrocarrier and allowed to air dry. Macrocarriers were then bombarded into the filters containing the chlorophyllous embryogenic cells of *B. gracilis*. The distance between the rupture membrane and the flying disk was 1.2 cm, and the macrocarrier traveled another 1.2 cm before impacting the steel-stopping screen. The filter disks containing the prestressed cells were then centered onto the Petri dishes, placed 7 cm from the launch point, and bombarded once at 800 psi. The sample chamber was evacuated to 0.04 atm before the gas acceleration tube was pressurized with helium. As part of our genetic transformation protocol (Aguado-Santacruz et al. 2001), the paper filters supporting the bombarded embryogenic cells were maintained for 3 d more on the same osmotic medium following particle bombardment. Fifteen Petri dishes were bombarded with each biolistic system. As controls, the same number of filters was bombarded with tungsten particles without DNA.

Selection of stably transformed lines and recovery of plants. After the 3-d postbombardment osmotic treatment on propagation medium containing 0.4 M mannitol but lacking antibiotic, the paper filter disks supporting the bombarded cells were transferred onto solid propagation medium containing 140 mg L^{-1} kanamycin and incubated at 30±1°C in white light provided by cold fluorescent lamps (photon flux=117 µmol s^{-1} m^{-2}). The kanamycin concentration was lowered to 120 mg L^{-1} 2 mo later. The cells were subcultured every 3 wk and maintained for another 6 mo in selective media. After this period, kanamycin-resistant lines were transferred to regeneration medium containing full-strength MS medium (Murashige and Skoog 1962), 3% sucrose, 2.5% phytagel (Sigma, St. Louis MO) but no antibiotic. The regenerated shoots were transferred for rooting to half-strength MS medium solidified with 2.5% phytagel and incubated at 30±1°C under contin-

uous cold fluorescent light (photon flux=117 μmol s^{-1} m^{-2}). Rooted plants were then transferred to pots, hardened off, and grown to maturity in a greenhouse.

In planta resistance to kanamycin. Resistance of regenerated plants established in the greenhouse was tested according to the procedure described in Aguado-Santacruz et al. (2001). Briefly, 30 d after transplantation of regenerated transgenic and control plants to pots in a greenhouse, they were sprayed every 2 d with 3 ml of a solution containing 150 mg L^{-1} kanamycin plus 0.1% sodium dodecyl sulfate (SDS). After 2 wk, the concentration of kanamycin was raised to 200 mg L^{-1} and applied daily for another 7 d. A final daily spraying of 250 mg L^{-1} of this antibiotic was applied to the plants for the next week.

DNA extraction. Harvested tissues were frozen in liquid nitrogen and kept at −80°C until RNA and DNA extractions were carried out. Total genomic DNA was prepared from kanamycin-resistant and untransformed control plants using a modification of the protocol described by Lopes et al. (1995).

PCR analysis. For polymerase chain reaction (PCR) analysis, 100 to 150 and 10 ng were used for genomic and plasmid DNA amplifications, respectively, in 25 μl reactions. Primers 5′-GCC GGA CTA AAC GTC GCC GGC-3′ and 5′-TCC CGT AGG TTT GTT TCG AAA-3′ were designed for amplifying an internal 250-bp fragment of the *rolA* gene, while primers 5′-TAT TCG GCT ATG ACT GGG CA-3′ and 5′-GCC AAC GCT ATG TCC TGA TA-3′ were used for amplifying a 620-bp internal fragment of the *nptII* gene. PCR reactions were carried out using a thermocycler Palm-Cycler (Corbett Research, Downers Grove, IL) for 35 cycles in which the reaction temperatures were as follows: denaturation at 95°C (2 min), annealing at 58°C (60 s), and extension at 72°C (60 s). The 25-μl reaction volumes contained: 1X PCR buffer, 0.25 mM deoxynucleoside triphosphates, 2 mM $MgCl_2$, 0.20 μM primers, and 2.5 U of *Taq* polymerase. The amplification products were electrophoresed in 1% agarose/ethidium bromide gels.

Southern blot analysis. Southern blotting and hybridization were carried out following the manufacturer's manual for the DIG system (Boehringer, Mannhein, Germany). Total plant DNA (30 μg $A_{260/280}$) from putative transformants and wild-type plants was digested overnight at 37°C using 30 U of *Bam*HI, separated in a double-thick 0.8% agarose gel and transferred to nylon membranes (Hybond N+, Amersham Biosciences, Piscataway, NJ). The filters were prehybridized at 65°C in 5X SSC, 1% blocking reagent (Boehringer, Mannhein, Germany), 0.01% SDS, and 0.1% lauroylsarcosine for 4 h and then hybridized overnight at 65°C in the same buffer. Digoxigenin-labeled probes from *rolA* (250 bp) and *nptII* (620 bp) genes were prepared by PCR using 15 ng of plasmidic DNA as template. Filters were sequentially washed in 2X SSC, 0.1% SDS for 5 min at room temperature, 1X SSC, 0.1% SDS at 65°C for 15 min, and 0.5X SSC, 0.1% SDS at 65°C for 5 min. Filters were visualized by autoradiography using Biomax films (Kodak, Rochester, NY).

Northern blot analysis. Total RNA was extracted from adult plant tissues according to López-Gómez and Gómez-Lim (1992) with some modifications. Fifteen milligrams of total RNA were electrophoresed in 1.2% formaldehyde–agarose gels immersed in MOPS buffer. After transfer to a positively charged nylon membrane (Hybond N+, Amersham Biosciences, Piscataway, NJ) in 10X SSC, blots were hybridized to a PCR digoxigenin-labeled *rolA* probe. Northern blot filters were visualized by autoradiography using Biomax films (Kodak, Rochester, NY).

Physiological performance of rolA-transformed plants. Once the transgenic status of the *rolA* transgenic blue grama plants was confirmed by PCR, Southern blot, and Northern blot analyses, these transgenic, as well as control plants, were maintained and increased during 4 mo in a greenhouse under optimal growth conditions. After this period, the morphology and physiology of *rolA*-transformed plants were compared to control plants. To accomplish this comparison, clonal bunches from every transgenic line and from untransformed plants were dissected, and 10- to 15-cm-tall one-tillered plants were then selected for evaluation. Three of these dissected plants were transplanted into individual 6-L-capacity pots containing 8 kg of sandy soil; ten pots (repetitions) were considered per transgenic or control (wild-type) line. Transplanted tillers were then permitted to grow for 1 mo, applying 200 ml of Long Ashton nutrient solution (Hewitt and Smith 1975) every 15 d and watering once a week to field capacity, which was specifically determined for our soil substrate according to Daubenmire (1974). Following this establishment period, an experimental homogenization was carried out by clearing the containers to one plant per pot, selecting even-sized plants. After this clearing process (day 0), the following variables were determined in the plants weekly during 4 mo: plant height (measured at the top of the largest leave), basal diameter (measured at soil level), and number of tillers and leaves per plant (bunch). Additionally, three evaluations of biomass gain (in terms of dry weight) were carried out through the experiment; the first sampling took place 2 mo after the clearing procedure and two more monthly evaluations were carried out during the subsequent 60 d. For shoot growth determinations, all plants were cut 2 cm above the soil level. At the end of the experiment

(119 d after the clearing process), the plants were extracted from the pots, their roots thoroughly washed with tap water, and evaluated for all the above-mentioned variables plus root dry weight. For determinations of shoot and root dry weights, the plant material was maintained in an oven at 70°C for 2 d until constant weights were reached. A completely randomized experimental design was used for analyzing differences among transgenic and control plants in morphological and productive variables. Duncan's tests were utilized to separate mean differences where F values showed significant differences ($p<0.05$; Steel and Torrie 1986).

Results

Fast growth and development of high chlorophyll content in selective media were instrumental in identifying positive lines, a criterion previously demonstrated to be a reliable indicator for genetic transformation of the chlorophyllic cell line of *B. gracilis* when kanamycin is utilized as a selective agent (Aguado-Santacruz et al. 2001). Putative transgenic lines appeared as green or green-yellowish points within yellow, light yellow, yellowish-white, or white cell masses within 4 d after transfer to selective medium containing 140 mg L^{-1} kanamycin. These green spots were then individually subcultured into fresh propagation medium containing 140 mg L^{-1} kanamycin. Some bombarded cell lines that initially appeared as greenish-yellow spots turned green with subculturing (after approximately 3–4 mo).

After maintaining the cells in selective media with 120 mg L^{-1} kanamycin for six more months, we obtained a higher number of kanamycin-resistant lines from blue grama cells bombarded with pLBR29 (containing the *rolA* and *nptII* genes) than from cells bombarded with tungsten particles alone (Table 1). From these kanamycin-resistant lines, only five lines from cells bombarded with PIG and three bombarded using the PDS-1000 system were able to develop high chlorophyll contents. When these eight green lines were transferred to regeneration and, subsequently, to rooting media, only four lines possessed regeneration capacity (RA-1, RA-2, RA-3, and RA-5 transgenic lines). In our previous experience with these cells, we found that only *nptII*-transformed blue grama chlorophyllous cells are capable of retaining their chlorophyll content under kanamycin-based selection schemes and regenerating into whole plants. Likewise, our previous transformation efficiencies (Aguado-Santacruz et al. 2002) using the PIG system and a construct containing the *nptII* gene (up to 9.5 positive lines per bombarded disk) are in stark contrast with the results obtained in the present study using the pLBR29 plasmid with only 0.33 and 0.20 positive lines per bombarded disk for the PIG and PDS biolistic systems, respectively.

In vitro phenotype of putative transgenic plantlets regenerated from antibiotic resistant lines. Regenerating somatic embryos from the putative transgenic lines RA-1, RA-2, RA-3, and RA-5 were heavily indumented (Fig. 2*a*, *b*), especially those derived from RA-5. These "hairy embryos" were not observed in wild-type embryogenic cell cultures regenerated under nonselective media (Fig. 2*c*) or in previous transformation experiments using *nptII* as a selective marker in our chlorophyllic system (Aguado-Santacruz et al. 2002). Likewise, hyperplasic roots were sometimes observed in regenerating pLBR29-bombarded chlorophyllic cell cultures (Fig. 2*d*). The transgenic status of plants regenerated from these kanamycin-resistant "hairy embryo"-developing lines was subsequently evaluated by PCR, Southern blot, and Northern blot analyses.

Molecular analysis of putative rolA-transformed lines of Bouteloua gracilis. To determine whether kanamycin-resistant regenerated plants were transgenic, a preliminary screen was carried out by PCR with primers designed to amplify internal fragments of *nptII* (620 bp) and *rolA* (250 bp) genes in adult plants that were regenerated from "hairy embryos" (Fig. 3). The amplified products revealed the presence of the expected bands in putative transformants

Table 1. Putative *rolA* transgenic lines of blue grama recovered after bombardment of the "TADH-XO" cells with two different biolistic systems

Lines after selection	PIG		PDS-1000	
	rolA-bombarded	Control	*rolA*-bombarded	Control
Kanamycin-resistant[a]	19	9	14	7
Green	5	0	3	0
Regenerating into whole plants	3	0	1	0

For each condition, 15 filter disks containing each one approximately 2 g FW cells were bombarded

[a] Determined after 8 mo in selection media containing 120 mg L^{-1} kanamycin

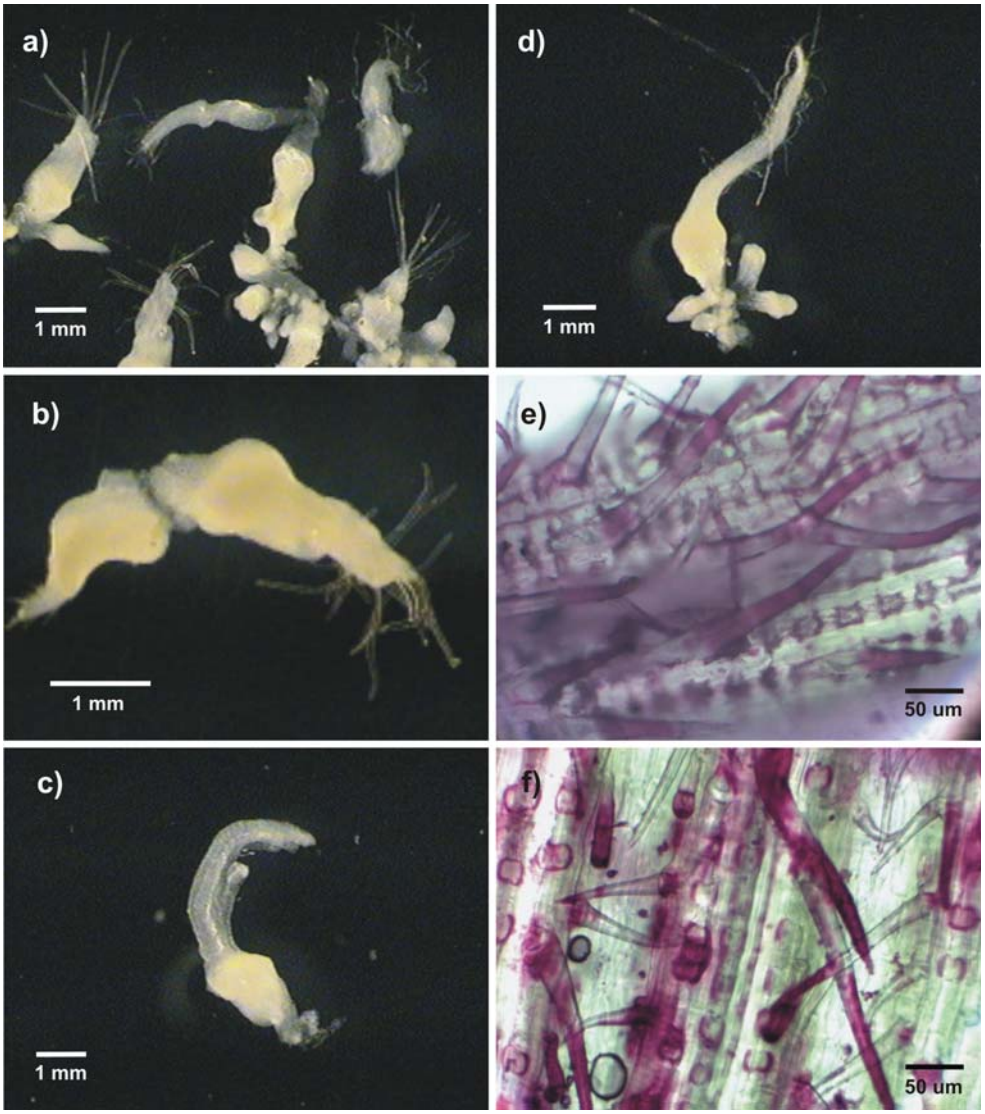

Figure 2. *In vitro* regeneration of transgenic and wild-type somatic embryos from *B. gracilis* chlorophyllous cells. *a*, Different development phases of 120 mg L^{-1} kanamycin-resistant, indumented, putative *rolA*-transformed embryos; *b*, close-up of two "hairy embryos" developing distinctive pubescence at their apical region; *c*, wild-type, glabrous embryo; *d*, hyperplasic root formed in embryogenic cultures bombarded with pLBR29; *e*, size of hairs developed in leaves of adult plants transformed with pLBR29; *f*, normal hairs developed in control plants.

(RA-1, RA-2, RA-3, and RA-5 lanes) and in positive controls (plasmidic DNA, lanes C+) for both *nptII* (approximately 620 bp) and *rolA* (approximately 250 bp) genes (Fig. 3); these bands were absent in wild-type control plants regenerated under nonselective conditions (lanes C−). Thus, an apparent integration of both genes was achieved in these plants, explaining the "hairy" kanamycin-resistant phenotype of *B. gracilis* somatic embryos.

To confirm the integration of the *rolA* transgene in the *B. gracilis* genome, Southern blot hybridization analysis was carried out on plants that were positive by PCR analysis. For this, total DNA from PCR-positive plants and untransformed control plants was digested with *Bam*HI, separated in a 0.8% agarose gel, transferred to a nylon membrane, and hybridized against digoxigenin-labeled probes from *nptII* (620 bp) and *rolA* (250 bp) genes. Because *nptII* and *rolA* genes contained in pLBR29 lack any *Bam*HI site (Fig. 1), high-molecular-weight bands are expected when DNA restricted with this enzyme is hybridized to digoxigenin-labeled probes from either gene. As observed in Fig. 4, high-molecular-weight signals were obtained when *nptII* or *rolA* probes were hybridized against the immobilized DNA. Every transgenic line showed a characteristic hybridization pattern, indicating that independent transformation events occurred. One to four independent insertion events could be distinguished among the different transgenic lines. For *nptII* hybridization, signals between approximately 3.0 and 4.3 kb were evident, while the *rolA* probe hybridized to DNA fragments ranging between 2.5 and 4.5 kb (Fig. 4).

When the relative accumulation of *rolA* mRNA in transgenic plants was estimated by northern analysis as described in "Materials and Methods" section (Fig. 5), it was observed that large amounts of *rolA* mRNA accumulated in green tissues of the RA-5 transgenic line (track 4 in Fig. 5). This line was characterized by an accentuated "hairy embryo" phenotype as described in the next section. The transcripts detected were approximately 650 bp, which

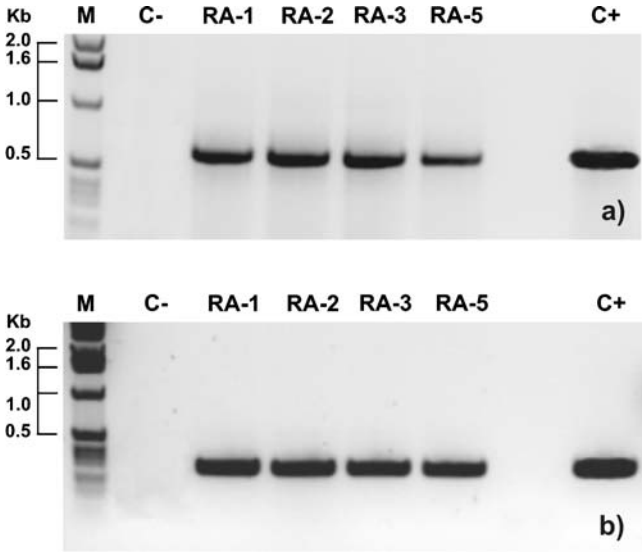

Figure 3. PCR analysis of kanamycin-resistant putative transgenic plants of *B. gracilis*. *a* Amplification of a 620-bp fragment from the *nptII* gene. *b* Amplification of a 250-bp fragment from the *rolA* gene. *Lane M* 1 kb molecular marker, *lane C−* untransformed control plant, *lane C+* positive control (pLBR29 DNA), *RA-1*, *RA-2*, *RA-3*, and *RA-5* plants regenerated from independent putative transgenic lines of *B. gracilis*.

is consistent with the size of the *rolA* mRNA reported by Spena and Langenkemper (1997). The variation between the results obtained for mRNAs of the individual transformants analyzed could be attributed to copy number and/or positional effects of the randomly inserted *rolA* gene.

Morphology and development of the rolA transgenic plants. One month after transfer to soil conditions in a greenhouse, five plants from every transgenic line and wild-type plants were assayed for resistance to kanamycin by applying 150 mg L^{-1} of this antibiotic on the plant foliage. As observed previously (Aguado-Santacruz et al. 2001), foliar application of kanamycin caused necrosis and drastically reduced the growth and tillering in non-transformed plants compared to transgenic plants. The indumentation previously observed in *rolA*-bombarded, regenerating somatic embryos *in vitro* was conserved in the leaves of adult plants as an abundant hairiness (Fig. 2*e*, *f* and Table 2). These characteristics were again especially pronounced in the high *rolA*-expressing transgenic line RA-5 (Table 2). The hair length of plants regenerated from this transgenic line averaged 0.1322 mm; that of wild-type plants was statically lower, 0.0607 mm ($p<0.05$). Likewise, hair densities were statically higher ($p<0.05$) in RA-5 (58 trichomes per square millimeter) than in control plants (29 trichomes per square millimeter; Table 2). In addition, abnormal spike development and infertility were observed in all transgenic lines (not shown). Besides these alterations, no other evident morphological alterations, such as those observed by Lee et al. (2001) in *rolA*-transformed rice plants, *i.e.*, wrinkled, dark green leaves or twisted roots, were displayed by the regenerated *rolA*-transformed *B. gracilis* plants.

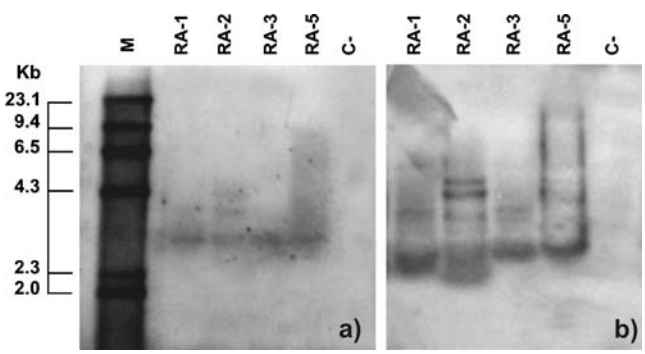

Figure 4. Southern blot analysis of PCR-positive, kanamycin-resistant, "hairy embryo"-derived plants of *B. gracilis* for *nptII* (*a*) and *rolA* (*b*) detection. *Lane M* λ *Hin*DIII molecular marker, *lane C−* untransformed control plant, *RA-1*, *RA-2*, *RA-3*, and *RA-5* plants regenerated from independent putative transgenic lines of *B. gracilis*.

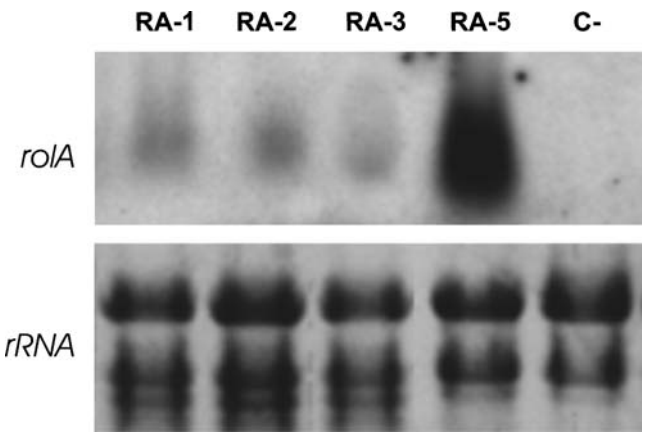

Figure 5. Northern hybridization analysis of *rolA* gene expression in transgenic plants of *B. gracilis*. Hybridization analysis was performed on fully developed plants. *Lane C−* untransformed control plant, *RA-1*, *RA-2*, *RA-3*, and *RA-5 rolA*-transformed plants regenerated from independent transgenic lines of *B. gracilis*. Fifteen-microgram aliquots of total RNA were loaded per lane and hybridized against a PCR digoxigenin-labeled *rolA*-coding region probe. Ribosomal RNA was utilized as loading control.

Physiological performance of rolA transgenic plants in the greenhouse. The physiological performance of the transgenic plants was compared to that of control plants under greenhouse conditions. Compared to RA-2 transgenic and control lines, plant height and basal diameter were consistently higher in RA-1, RA3, and RA-5 transgenic lines through all experiments with differences being statistically significant ($p<0.05$) from RA-2 and control plants at 63, 91, and 119 d after experiment initiation (Table 3). Differences in plant height increased as the plant

Table 2. Trichome length and density in *rolA*-transformed plant lines of blue grama grass

Variable	Plant lines				
	Control	RA-1	RA-2	RA-3	RA-5
Length (mm)	0.0607a	0.0969b	0.0807ab	0.0895b	0.1322c
Density (number per square millimeter)	29a	42b	36ab	46b	58c

Different *letters* indicate significant differences ($p<0.05$) among plant lines

development progressed. At the first evaluation (2 mo after the initiation of the experiment), plants from RA-1, RA3, and RA-5 transgenic lines were 58.1, 57.5, and 59.9 cm tall, respectively; plants from the RA-2 and control plants were only 45.3 and 43.0 cm, respectively. However, when only 1 mo (day 91) had elapsed since plant clipping, heights registered in RA-1, RA-3, and RA-5 plants averaged 26.7 cm, while those observed in RA-2 and control plants were around 21 cm (Table 3). In contrast to plant height, basal diameter differences among the different transgenic and control lines gradually increased from day 0 to day 119. At the end of the experiment, plants from the RA-5 line showed a basal diameter almost 1.6-fold higher than that of RA-2 and control plants ($p<0.05$; Table 3). To explain these results, tiller and leaf numbers were quantified in transgenic and control plants. As expected, tillering rates were increased in transgenic lines with respect to control plants. At day 91 and as a result of the clipping treatment, transgenic and control plants experienced tiller mortality that was especially pronounced in the RA-2 line (not shown). As opposed to control and RA-2 plants, RA-1, RA-3, and RA-5 plants recovered and resumed tiller

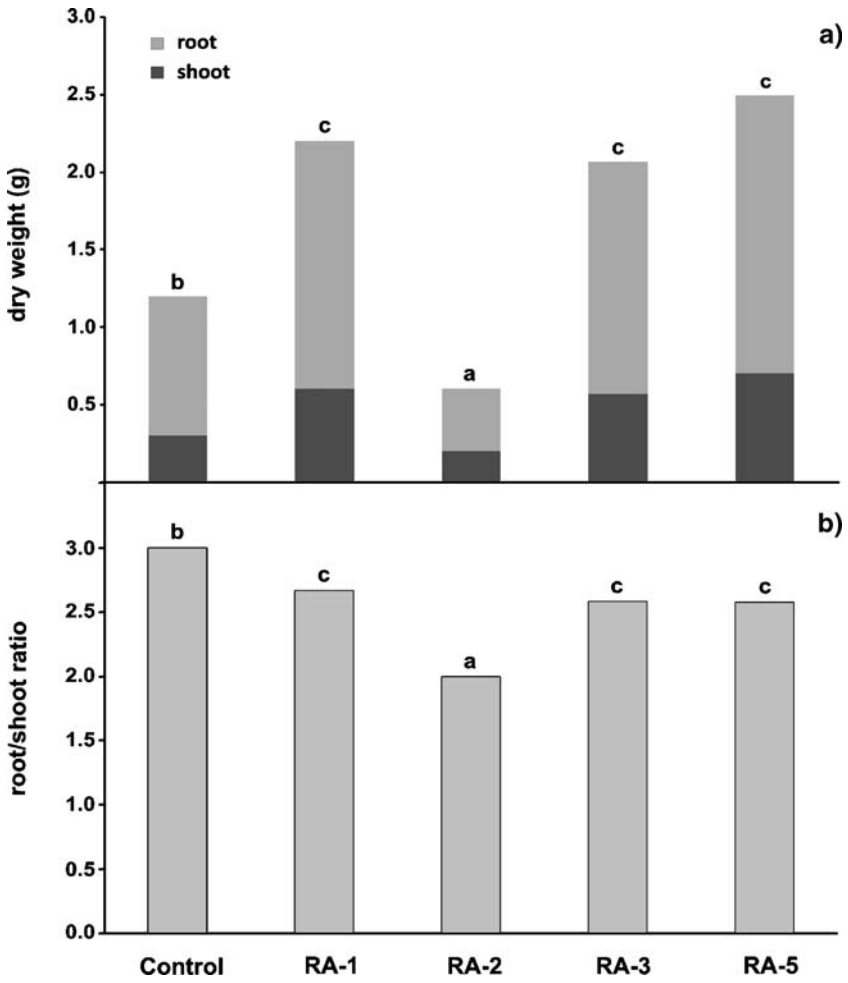

Figure 6. Biomass production (*a*) and root/shoot ratios (*b*) in control and transgenic plants. Data are means of 15 replicates from two independent experiments. *Bars showing different letters* indicate significant differences ($p<0.05$) among plant lines.

Table 3. Physiological performance at three sampling dates of *rolA*-transformed *B. gracilis* plants

Variable	Days	Plant lines				
		Control	RA-1	RA-2	RA-3	RA-5
Plant height (cm)	63	45.3a	58.1b	43.0a	57.5b	59.9b
	91	20.5a	27.2b	22.1a	26.8b	26.0b
	119	28.3a	39.3b	29.4a	36.1b	37.1b
Basal diameter (cm)	63	1.3a	1.8b	1.6ab	1.8b	2.2c
	91	1.7a	2.3b	1.8a	2.0ab	2.7c
	119	2.3a	3.0b	2.3a	2.7b	3.5c
Number of tillers/plant	63	11.9a	17.7b	18.2b	16.0b	19.0b
	91	19.4a	27.8b	26.7b	26.2b	33.4c
	119	17.3a	26.7b	18.8a	27.4b	29.4b
Number of leaves/plant	63	35.0a	44.7b	44.3b	43.0b	48.9b
	91	36.5a	51.8b	46.8b	49.4b	59.6c
	119	33.9a	46.5bc	30.3a	44.9b	50.7c

Different *letters* indicate significant differences ($p<0.05$) among plant lines

production 7 d later (not shown). At day 119, plants from RA-1, RA-3, and RA-5 transgenic lines produced 26.7, 27.4, and 29.4 tillers, respectively; RA-2 and control plants generated only 18.8 and 17.3 tillers, respectively (Table 3). Similarly, at this sampling date, leaf numbers were only 33.9 and 30.3 for control plants and RA-2 plants, respectively; RA-1, RA-3, and RA-5 transgenic lines produced a significantly higher number of leaves per plant (46.5, 44.9, and 50.7, respectively; Table 3). All of these physiological variables correlated well with overall productivity measured as plant biomass (dry weight gain). Plants from RA-1, RA-3, and RA-5 transgenic lines attained the highest dry weights in both root and shoot components at the two intermediate sampling dates (not shown) and at the end of the experiment, when dry weights of these plants were almost double those obtained by control plants ($p<0.05$; Fig. 6*a*). Conversely, and in association with all the previous physiological variables analyzed, RA-2 plants attained the lowest dry weights from all plants tested. Finally, the root/shoot ratios calculated for transgenic and control plants indicated that the increased plant biomass observed in the *rolA*-transformed plants was mainly related to shoot growth (Fig. 6*b*).

Discussion

The effects of transforming dicot plants with the *rol* genes of *A. rhizogenes* are well documented. Although they appear to act in a complex manner that impede establishment of clear and generalized action patterns of these genes, the contributed experimental evidence shows that the *rol* genes may be interesting candidates for biotechnological applications. Within these genes, *rolA* has emerged as a stimulator of growth and secondary metabolism (Bulgakov 2008).

Transformation of tobacco with the *rolA* gene driven by its own promoter results in normal-sized plants with wrinkled leaves and reduced internode distances, condensed capsules, abnormal stigma size, and larger flowers (Schmülling et al. 1988), whereas transgenic plants containing the *rolA* driven by the CaMV 35S promoter show stunted growth with dark, wrinkled leaves (Dehio et al. 1993). The effects of *rolA* transformation in *Solanum dulcamara* include increased stature, reduced internode distance, and wrinkled leaves (McInnes et al. 1991). Similar results are reported for tomato plants transformed with the *rolA* gene (Van Altvorst et al. 1992).

In contrast to dicots, the well-recognized inability of wild-type strains of *Agrobacterium* to transform grasses, particularly cereals, has hampered the analysis of the morphogenetic activities of *rol* genes in monocotyledonous plants (Chriqui et al. 1996). Development of *Agrobacterium*-free genetic transformation methods, such as biolistics, has allowed plant scientists to overcome this barrier and establish the effects of particular genes in new genetic contexts.

A major advance toward exploring the traits associated with *rol* gene expression in the monocots was reached by Lee et al. (2001) in rice. These rice plants were transformed with a construct containing the *rolA* gene under the control of a double 35S promoter using PEG-mediated protoplast transformation. Some alterations detected in these rice transgenic plants resembled those reported previously for dicots, *i.e.*, dark green, wrinkled leaves. Other alterations seem to be specific to this cereal, such as tumor-like structures with emerging roots on the leaves, downward-pointing leaf tips, callus-like outgrowths on the upper leaf surface, root emergence at the collar region of the leaves,

corkscrew-like and twisted primary roots, and extensive root hair development. Additionally, in comparison to wild-type control plants, *rolA*-transformed rice lines showed (a) reduced root system, leaf, and panicle size, (b) smaller stature, and (c) fewer primary branches and spikelets per panicle. An effect on reproductive behavior could not be confirmed because both *rolA* transgenic as well as protoplast-derived control plants were infertile.

In this work, we have utilized a forage grass known as blue grama, *B. gracilis*, as a model for *rolA* transformation and analyzed some of the effects of this transgenesis. First, the results presented here confirm our previous findings on the efficiency of the *nptII* gene as a selectable marker for genetic transformation of the blue grama "TADH-XO" chlorophyllic cell line because all kanamycin-resistant green lines were transgenic. The lower transformation efficiencies in the experiments carried out in this work using plasmid pLBR29 containing the *rolA* gene driven by a double CaMV 35S promoter (up to 0.3 positive lines per bombarded filter), contrast with previous efficiencies obtained with a plasmid containing a GUS::NPTII fusion protein under the control of the same promoter (up to 9.7 positive lines per bombarded filter; Aguado-Santacruz et al. 2002). It is probable that pleiotropic effects linked to *rolA* expression could interfere with the processes involved in chlorophyll development in our embryogenic cell line when it is subjected to the kanamycin selection scheme; chlorophyll accumulation is utilized as an efficient visual marker for the detection of putative transformant lines in this system (Aguado-Santacruz et al. 2002). In the rice work by Lee et al. (2001), similar results were obtained with only 13 of 461 G418 (Geneticin)-resistant colonies producing a single green plant. Technical problems associated with the PEG-mediated protoplast transformation method used by these authors could also have contributed to this low regeneration efficiency.

Once the transgenic lines of *B. gracilis* could develop enough chlorophyll to be identified as *nptII* positive (see Aguado-Santacruz et al. 2002), a second phenotypic marker, not reported previously for *rol* gene transgenesis, was then very useful for identifying transformation events linked to the *rolA* integration; namely, the development of a very conspicuous indumentation in regenerating somatic embryos. This embryo phenotype had not been previously reported for genetic transformation of dicots or monocots and constitutes a new useful visual phenotypic marker for the early identification of *rolA* transformation events. It would be important to test in systems that are analogous to ours, such as *Lolium* or *Festuca*, for which the biotechnological frameworks required for genetic transformation are always one step ahead, whether indumentation developed in response to *rolA* expression can also be utilized as a reliable and useful visual marker for the early identification of transgenic events using either the *rolA* or the rest of the *rol* genes, separately or combined.

Although RA-5 transgenic line displayed an exceptional level of *rolA* transcript and a pronounced "hairy syndrome," RA-1, RA-2, and RA-3 with similar levels of *rolA* transcripts showed different morphological and productive performance. The differences between phenotype strength and mRNA levels might occur because of post-transcriptional and post-translational regulation of the *rolA* transcript as presumed for individual *rolA* transgenic clones of apple by Xue et al. (2008). Alternatively, because *rolA* affects hormone metabolism (Maurel et al. 1991; Sun et al. 1991; Vansuyt et al. 1992; Dehio et al. 1993; Schmülling et al. 1993; Prinsen et al. 1994) and the result of growth regulators action is concentration-dependent and balance-dependent (Salisbury and Ross 1992), it would be expected that subtle thresholds for the *rolA* action, hardly established or inferred from the mRNA levels, affected differently the response of plants to this protein.

Leaf hairiness in adult plants was maintained for at least 1 yr after the initial establishment of T0 plants in soil. However, this characteristic has not been stably maintained because clonal tillers derived from the initial transformed shoot showed reversion to the original glabrous leaf phenotype. Nevertheless, as indicated by the molecular analysis carried out, these plants have preserved the *rolA* genetic information. Reversion via transcriptional inactivation (hypermethylation) of aberrant plants transformed with the *rol* genes to the wild-type phenotype has been reported previously in *Nicotiana glauca* in an age-dependent manner (Sinkar et al. 1988b). Inactivation of the *tmr* gene of the nopaline-type Ti plasmid in transgenic *N. tabacum* is also mediated through hypermethylation (Amasino et al. 1984).

Previous studies have confirmed the potential of *rol* genes for crop improvement (Strobel and Nachmias 1985; Strobel et al. 1988; Lambert and Tepfer 1991; Rugini et al. 1991; Stomp et al. 1993; Frugis et al. 1995; Van der Salm et al. 1997; Tzfira et al. 1999). Although transformation of *B. gracilis* with the *rolA* of *A. rhizogenes* resulted in increased biomass gain in three of the four transgenic lines, it was also evident that, as previously reported for rice (Lee et al. 2001) and tobacco (Carneiro and Vilaine 1993), *rolA* transformation resulted in deficient root system development.

Interestingly, *rolA* expression in dicot plants has been associated with reduced apical dominance (Sinkar et al. 1988b; McInnes et al. 1991) and, consequently, with increased branching, while in forage grasses, any factor resulting in apical dominance breakage will be conducive to increased tillering (Vallentine 1990) and thus to increased shoot biomass. Control of tillering is a very important biotechnological issue in monocots because of its potential application for improving their agronomic performance. Li et al. (2003) have emphasized the potential improvement of

grain production in rice by modifying a single gene (*MOC1*) related to tillering control, while the role of strigolactones (compounds thought to be derived from carotenoids) in the control of shoot branching has been recently recognized (Gomez-Roldan et al. 2008; Umehara et al. 2008). In forage grasses, the control of apical dominance for increased biomass production has been well recognized for a long time (Vallentine 1990). Because our data show a relation of increased biomass to accelerated tillering rate, a new feasible biotechnological application for agronomic improvement of forage grasses is established in this work by transforming forage grasses with the *rolA* gene of *A. rhizogenes*. It is expected that physiological studies currently under way contribute further evidence on this possibility.

We expect that future transformation studies of cereals and forage grasses employing the *A. rhizogenes* genes will provide additional evidence to explain the effects and mechanisms of *rol* gene action in plants.

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