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CROP BREEDING AND APPLIED BIOTECHNOLOGY

ARTICLE

Agrobacterium-mediated genetic transformation of a tropical elite maize line

Crop Breeding and Applied Biotechnology
17: 133-140, 2017
Brazilian Society of Plant Breeding.
Printed in Brazil
http://dx.doi.org/10.1590/1984-70332017v17n2a20

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Abstract: *The efficiency of maize transformation mediated by Agrobacterium tumefaciens is influenced by various factors. The aim of this study was to test the effect of different concentrations of N6 salts (50% – half strength, and 100% - full strength) in the infection and cocultivation media on genetic transformation efficiency of the L3 tropical elite maize line. Immature embryos were transformed via A. tumefaciens harboring the binary vector pTF102 containing the genes uidA and BAR under control of the CaMV35S promoter. The efficiency of the transgenic events produced was 3% for full strength and 1.1% for half strength N6 salts. Although under a lower concentration of salts, GUS expression was stronger; under this concentration, regeneration was less efficient. Thus, the results showed that the presence of 100% N6 salts in the infection and cocultivation media favored genetic transformation of the L3 maize inbred line mediated by A. tumefaciens.*

Key words: *Immature embryos, salt concentration, somatic embryogenesis, Zea mays.*

INTRODUCTION

Brazil produced 84.7 million tons of maize on 15.7 million hectares in 2015 (Conab 2016) and it is estimated that 80% of the maize harvested is genetically modified. So far, no Brazilian company has implemented a pipeline for transformation and commercial release of tropical maize lines. Governmental and private agricultural institutions in Brazil have been conducting investigations related to the adaptation of maize to biotic and abiotic stresses aiming at an increase in yield, and genetic transformation of tropical maize lines may have a major impact on the development of new products based on this knowledge. One of the main obstacles to application of this technology to tropical maize lines is the selection of germplasm responsive to regeneration in tissue culture and infection by *Agrobacterium*.

The regeneration pathway most used for transgenic maize cells is somatic embryogenesis, which was first described by Green and Phillips (1975) using immature maize embryos as explants. Maize embryogenic cultures have two predominant forms of callus, Type I and Type II, which differ primarily in their regeneration efficiency over an extended time in a culture medium. Although both calli are capable of plant regeneration, cultures formed by the Type II callus grow faster; can be maintained for a longer period of time, and form a larger

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Received: 08 July 2016
Accepted: 01 December 2016

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number of somatic embryos. These characteristics favor the selection and regeneration of transgenic plants (Johnson and McCuddin 2009). The occurrence of the Type II friable embryogenic callus is not so common, only a limited number of maize genotypes are able to express this phenotype, especially the hybrid Hi-II (Armstrong and Green 1985). Because of this characteristic, Hi-II is one of the most used genotypes in genetic transformation protocols. However, it has low agronomic performance (Que et al. 2014). The transgene can be transferred to elite varieties using backcrosses; but this process is long, and unwanted traits can be transmitted together with the gene of interest.

Regeneration and transformation are largely influenced by the explant type and the composition of the culture medium (Armstrong and Green 1985, Songstad et al. 1991, Bohorova et al. 1995). To date, immature zygotic embryos are the explant with the highest regeneration efficiency, and competence for infection by *A. tumefaciens* and generation of transgenic plants (Frame et al. 2006, Vega et al. 2008).

The N6 (Chu et al. 1975) and MS (Murashige and Skoog 1962) salts are widely used for *in vitro* cultivation and genetic transformation of maize (Ishida et al. 1996, Frame et al. 2006, Vega et al. 2008, Li et al. 2015). Embryogenic calli are normally formed when immature zygotic maize embryos are cultured in these basal media supplemented with auxins, such as 2,4-D or Dicamba (Green and Phillips 1975, Armstrong and Green 1985, Frame et al. 2006, Petrillo et al. 2008).

The salt content of the culture medium appears to be an important factor capable of influencing the efficiency of transformation mediated by *Agrobacterium*. Although the transformation improvement mechanism is not clear, the use of low salt contents through the infection and cocultivation stages has been shown to improve the transfer of T-DNA (Fry et al. 1987, Cheng et al. 1997, Armstrong and Rout 2001, Zhang et al. 2002, Paz et al. 2004, Vega et al. 2008).

An increase in T-DNA transfer efficiency was also achieved by supplementation of the infection and cocultivation media with antioxidants or anti-necrotic mixtures. Cysteine, dithiothreitol (DTT), polyvinyl pyrrolidone, and ascorbic acid (Perl et al. 1996, Olhoft and Somers 2001, Paz et al. 2004, Frame et al. 2006, Mishra et al. 2014) are antioxidants that have been used to increase the potential of *Agrobacterium* to infect tissues by inhibiting the antimicrobial exudates produced by plant cells during the infection and cocultivation stages and/or by preventing the occurrence of necrosis in the plant tissue (Gupta 2010).

The selection of genotypes to obtain transgenic maize plants focuses on *in vitro* regeneration capacity and the possibility of infection by *Agrobacterium*. An ideal genotype for the production of commercial transgenic plants, in addition to the features mentioned above, must have excellent agronomic performance. The L3 tropical elite maize inbred line from Embrapa maize germplasm has all these desirable characteristics, and the aim of this study was to identify culture media that contribute most to its efficiency of transformation by *Agrobacterium tumefaciens*.

MATERIAL AND METHODS

Plant Material

The L3 tropical maize inbred line from Embrapa Milho e Sorgo (Embrapa Maize and Sorghum), located in the state of Minas Gerais, Brazil, was used for the transformation experiments. For embryo extraction, ears pollinated for 10-15 days were surface sterilized in half-strength liquid commercial bleach (2.5% sodium hypoclorite) and 0.01% Tween 20 for 30 minutes and then rinsed three times with sterile distilled water. In all experiments, immature embryos from 1.5 to 2.2 mm from the same ear were equally distributed among treatments to minimize variation.

Genetic construct and *Agrobacterium tumefaciens* preparation

The *A. tumefaciens* used was the disarmed strain EHA101 (Hood et al. 1986) harboring the binary plasmid pTF102 (Paz et al. 2004). The bacterial resistance marker in pTF102 is spectinomycin. This plasmid also contains the phosphinothricin acetyltransferase (BAR) gene as a plant selection marker and the reporter gene *uid*A, both controlled by the CaMV35S promoter. The *uid*A gene contains an intron to prevent its expression in *Agrobacterium* cells (Paz et al. 2004).

A. tumefaciens was cultivated in Yeast Extract Peptone (YEP) solid medium (5 g L^{-1} yeast extract, 10 g L^{-1} peptone, 5 g L^{-1} NaCl, 15 g L^{-1} Bacto-agar, pH 6.8) containing 100 mg L^{-1} spectinomycin and 50 mg L^{-1} kanamycin (for EHA 101) for three days at 19 ^oC. Two hours prior to transformation, *Agrobacterium* was resuspended at OD_{550} 0.3 to 0.4 in two different

Table 1. Culture media used for genetic transformation of immature embryos from the L3 tropical inbred maize line mediated by *Agrobacterium tumefaciens*

Medium	Composition
Infection Medium 1[1]	*1X N6[(3)] salts* and vitamins; 68.4 g L^{-1} sucrose; 36 g L^{-1} glucose; 0.7 g L^{-1} proline; 0.25 g L^{-1} cefotaxime; 1.5 mg L^{-1} 2,4-D; 100 µM L^{-1} acetosyringone; pH 5.2
Infection Medium 2[2]	*½X N6 salts* and vitamins; 68.4 g L^{-1} sucrose; 36 g L^{-1} glucose; 0.7 g L^{-1} proline; 0.25 g L^{-1} cefotaxime; 1.5 mg L^{-1} 2,4-D; 100 µM L^{-1} acetosyringone; 0.5 g L^{-1} 2-(N-Morpholino)ethanesulfonic acid (MES); 0.85 mg L^{-1} silver nitrate; pH 5.2
Cocultivation Medium 1[1]	*1X N6 salts* and vitamins; 30 g L^{-1} sucrose; 0.7 g L^{-1} proline; 0.25 g L^{-1} cefotaxime; 1.5 mg L^{-1} 2,4-D; 100 µM L^{-1} acetosyringone; 0.85 mg L^{-1} silver nitrate; *3 mg L^{-1} L-cysteine*; 3 g L^{-1} Phytagel, pH 5.8
Cocultivation Medium 2[2]	*½X N6 salts* and vitamins; 30 g L^{-1} sucrose; 0.7 g L^{-1} proline; 0.25 g L^{-1} cefotaxime; 1.5 mg L^{-1} 2,4-D; 100 µM L^{-1} acetosyringone; 0.5 g L^{-1} MES; 0.85 mg L^{-1} silver nitrate; *4 mg L^{-1} L-cysteine; 0.15g L^{-1} dithiothreitol*; 3 g L^{-1} Phytagel, pH 5.8
Resting	1X N6 salts and vitamins; 30 g L^{-1} sucrose; 0.7 g L^{-1} proline; 0.5 g L^{-1} MES; 0.85 mg L^{-1} silver nitrate; 0.25 g L^{-1} cefotaxime; 10 mg L^{-1} 2,4-D; 3 g L^{-1} Phytagel, pH 5.8
Selection I	1X N6 salts and vitamins; 30 g L^{-1} de sucrose; 0.7 g L^{-1} proline; 0.5 g L^{-1} MES; 0.25 g L^{-1} cefotaxime; 10 mg L^{-1} 2,4-D; 1.5 mg L^{-1} bialaphos; 3 g L^{-1} Phytagel, pH 5.8
Selection II	Selection I supplemented with 3.0 mg L^{-1} bialaphos
Maturation	1X MS[(4)] salts and vitamins; 60 g L^{-1} sucrose; 1.25 mg.L^{-1} $CuSO_4$; 3 g L^{-1} Phytagel; pH 5.8
Germination	MS salts and vitamins; 30 g L^{-1} sucrose; 3 g L^{-1} Phytagel; pH 5.8

[1] Frame et al. (2006); [2] Vega et al. (2008); [3] N6 (Chu et al. 1975); [(4)] MS (Murashige and Skoog 1962). All the reagents used were from Sigma Inc. unless otherwise specified.

infection media, infection medium 1 and infection medium 2 (Table 1), supplemented with 100 µM of acetoseringone and incubated at 22 °C and 100 rpm.

Genetic transformation of immature maize embryos of the L3 inbred line mediated by *Agrobacterium tumefaciens*

Genetic transformations of maize were performed using culture media 1 and 2 according to Frame et al. (2006) and Vega et al. (2008), respectively, with few modifications. All culture media are described in Table 1. Embryos were placed in 1.5 ml Eppendorf tubes containing 1.0 ml of infection medium (1 or 2) supplemented with 100 µM of acetosyringone and washed twice with the same medium. After removing the final wash, 1 ml of the *Agrobacterium* suspension, prepared using infection medium 1 or 2, was added for 5 min to tubes containing embryos. Then embryos from infection medium 1 were placed in cocultivation medium 1, and embryos from infection medium 2 were placed in cocultivation medium 2, with the axis in contact with the medium, and incubated at 20 °C for five days in the dark. After the incubation period, the embryos were transferred to a resting medium at 28 °C for two weeks in the dark. Resting, selection I and II, maturation, and germination media were the same for the embryos initially infected and cocultivated on medium 1 or 2.

Selection was initiated with the transfer of all embryos to selection medium I supplemented with 1.5 mg L^{-1} bialaphos (tripeptide L-phosphinothricyl-L-alanyl-L alanine) (Thompson et al. 1987) (Goldbio.com / St. Louis, MO, USA) for two weeks. Transfer to a new selection medium was repeated 2-3 times until the onset of embryogenic calli with vigorous growth. After that, these calli were transferred to selection medium II supplemented with 3 mg L^{-1} bialaphos, where they remained for 14 days. Selected calli were transferred to a maturation medium for 2-4 weeks at 27 °C. Mature embryos with a white opaque and dry appearance were grown on a germination medium for 2-4 weeks. Seedlings around 5 cm in length and with a well-developed root system were transferred to soil. After acclimatization, the plants were treated with 500 mg L^{-1} Finale© to ensure that all events in the greenhouse express the BAR gene. The presence of the BAR gene was also screened in T1 and T2 progenies by spraying germinated seedlings at 14 days post emergence with 500 mg L^{-1} Finale© .

GUS histochemical analyses

The expression of β-glucuronidase (*GUS*) was assessed by histochemical analyses (Jefferson et al. 1987) in 25 embryos after the cocultivation and resting stages and in 10 embryogenic calli after the selection process. Embryos or calli were immersed in a solution containing 1 mM 5-bromo-4-chloro-3-indolyl β-D glucuronide, 50 mM phosphate buffer pH 6.8, 20% methanol, and 1% Triton X-100, incubated at 37 °C for 16-24 hours. The presence or absence of blue staining was assessed using a Zeiss Axio Zoom V16 stereomicroscope. Embryos or calli that showed at least one blue spot were

considered positive for expression of the *uid*A gene.

Molecular analyses of transgenic events

The presence of the genetic construct was confirmed by PCR using genomic DNA from leaves of transgenic (T0) events (Saghai-Maroof et al. 1984) and primes for the BAR gene (5'AGA CAC AAC ATG GTC CC3' and 5'TGC ATC ACC GTC AAC CAC3'), which amplify a fragment of 407 bp. The 20 µL PCR reaction contained 0.1 U of Taq DNA Polymerase (Invitrogen /SP, Brazil), 2.5 mM $MgCl_2$, 200 µM dNTPs, 1X buffer, and 20 ng genomic DNA. Amplification was performed in a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA), using an initial denaturation at 94 ^{o}C for 2 minutes, 35 cycles of 94 ^{o}C for 20 seconds, 60 ^{o}C for 30 seconds, and 72 ^{o}C for 90 seconds, and a final extension at 72 °C for 5 minutes. The PCR products were visualized in 1% agarose gels, stained with 1:100 dilution GelRed Stain Nucleic Acid (Biotium / Uniscience /SP, Brazil).

To estimate the copy number of transgene insertions in the T0 events, a proprietary single copy transgenic maize plant MSALTSBL22 (Barros et al. 2011) and L3 line were used as a reference sample and negative control, respectively. The transgene used was the BAR gene (BAR *F*: 5'ACA GCG ACC ACG CTC TTG A-R3' / BAR *R*: 5'GCT CTA CAC CCA CCT GCT GAA3') and the reference was the *adh1* gene (*Adh1F*: 5' GTA ACA TGC TCC AGC ACT GCT ATT3' / *Adh1R*: 5'TCG TAT GAT GTG TTC AGC CAG ACT 3') (Ingham et al. 2001). The qPCR reactions were carried out in 10 µl reaction mixtures containing 50 ng template DNA, Fast SYBR Green Master Mix 1X (Applied Biosystems, Foster City, CA, USA), and 0.5 µM primers.

For expression data, total RNA was extracted from fresh leaves using the RNeasy Plant Mini Kit (Qiagen, São Paulo, SP, Brazil) according to manufacturer's instructions. First-strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, São Paulo, SP, Brazil). Expression of the *uid*A reporter (*GUSF*: 5' CGT GGC AAA GGA TTC GAT AA 3' / *GUSR*: 5' CTC TTC AGC GTA AGG GTA ATG 3') and BAR genes were assessed using a SYBR Green-based assay and actin gene as a reference gene (*ActF*: 5' TCC TGA CAC TGA AGT ACC CGA TTG3' / *ActR*: 5'-AGT TCG TTG TAG AAG GTG TGA TGC C3'). The qPCR reaction conditions were the same as described above.

All quantitative analyses were carried out in a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using the comparative CT method, ΔΔCT (Livak and Schmittgen 2001). Each biological sample was a bulk of leaves from ten T0 plants, with three technical replicates each.

Statistical analysis

The two media tested for infection and cocultivation were evaluated by comparing the percentage of *GUS* positive embryos or calli after cocultivation, resting, and selection, and by the number of transgenic callus and regenerated events obtained. The percentage of positive embryos or calli were calculated by multiplying the number of embryos or calli that had at least one blue spot by 100 and dividing this result by the total number of embryos or calli analyzed. The experimental design was completely randomized, with four replicates (200 embryos/replicate). For analysis of variance, the software R (R Foundation 2015) was used, and means were compared by the Tukey test at 5% probability.

RESULTS AND DISCUSSION

In the present study, the *uid*A gene was used as a reporter to compare the efficiency of gene transfer from *A. tumefaciens* to cells of immature embryos of the L3 tropical maize inbred line, after the cocultivation and resting stages, on media containing full (100%) and half (50%) strength N6 salts. Usually, conditions which allow a high level of expression of this reporter gene are associated with a high rate of production of transgenic events (Hiei at al. 1994, Petrillo et al. 2008, Vega et al. 2008).

After cocultivation with *Agrobacterium* EHA 101 harboring the binary vector pTF102, over 70% of L3 immature embryos were infected and capable of transiently expressing the *uid*A gene, regardless of the coculture medium used. A higher number of embryos was able to express the *uid*A gene when infection and cocultivation used full strength N6 salts (medium 1) in comparison with the half strength salts (medium 2) (Table 2). However, the histochemical assay revealed that *GUS* expression was stronger in the embryos maintained on half strength salts (medium 2) (Figure 1A and B). Although expression of the *uid*A gene has not been quantified, the expression pattern shown in Figures 1 A and B was always repeated in all maize transformations performed. Histochemical analysis comparing infection and

Table 2. Comparison between culture medium 1 and 2 used to transform immature embryos of the L3 tropical maize inbred line.

Parameter	Medium 1[1]	Medium 2[2]	CV (%)
Histochemical *uidA* test after			
Cocultivation (%)	89.66 a	74.00 b	5.21
Resting (%)	74.66 a	32.00 b	20.47
Selection II (%)	53.00 a	24.66 b	14.83
Transgenic events			
Number of resistant calli	18.75 a	9.50 b	16.79
Number of regenerated plants	12.25 a	4.00 b	53.35
Transformation efficiency (%)[3]	3.3	1.1	-

Means followed by the same letter in the line do not differ at 5% probability by the Tukey test

[1]100% and [2]50% N6 salt concentration
[3] Regenerated transgenic events x 100/Immature embryos.

cocultivation media 1 and 2 was conducted simultaneously and with the same solutions, thus ruling out any artifacts that could interfere with a differential expression of the reporter gene. Ke et al. (2002) report that transient expression of *GUS* in immature barley embryos infected with *A. tumefaciens* harboring the *uid*A gene increased 10 times when conducted in a medium containing 1/10 strength MS salts.

After the resting and selection II stage (Table 2), a higher number of embryos expressing *GUS* originated from medium 1, confirming that for the L3 maize line, the full strength N6 salt content was more efficient. Most of the embryos kept on half strength N6 salt (medium 2) developed poorly afterward. Vega et al. (2008), working on the transformation efficiency of Hi-II, reported that even though low strength salt (10%) improves gene transfer, it decreases the embryogenic response of the explant. The same developmental pattern was observed by Du et al. (2010); explants maintained on media supplemented with 10% and 30% salt displayed *GUS* staining, but most of them did not develop further and died. Even though half strength N6 salt enabled optimum T-DNA transfer, it had damaging effects on the embryogenic aptitude of immature maize embryos.

Another contrasting difference between these media is the presence of cysteine alone or cysteine plus DTT, in the infection and cocultivation media 1 and 2, respectively. The higher concentration of antioxidants present in medium 2 might have contributed to more effective *Agrobacterium* infection by inhibiting defense mechanisms such as the production of reactive oxygen species (ROS) by the immature embryos infected (Gupta 2010). This intensification in infection may have compromised embryo regeneration capability. Ishida et al. (2007) showed that immature embryos expressing higher levels of the *uid*A reporter gene after the cocultivation stage were not the best ones to regenerate transgenic maize plants.

A total of 106 calli that survived to the selection II stage grew rapidly and had a friable appearance and light yellow color (Figure 1C and D). Of this total, 61 were from the infection and cocultivation medium 1, and 45 from medium 2; all calli selected were transferred to the maturation medium. Maturation occurred unevenly within the period of 20 to 45 days, regardless of the media used for infection and cocultivation. Only 42.62% and 20% of

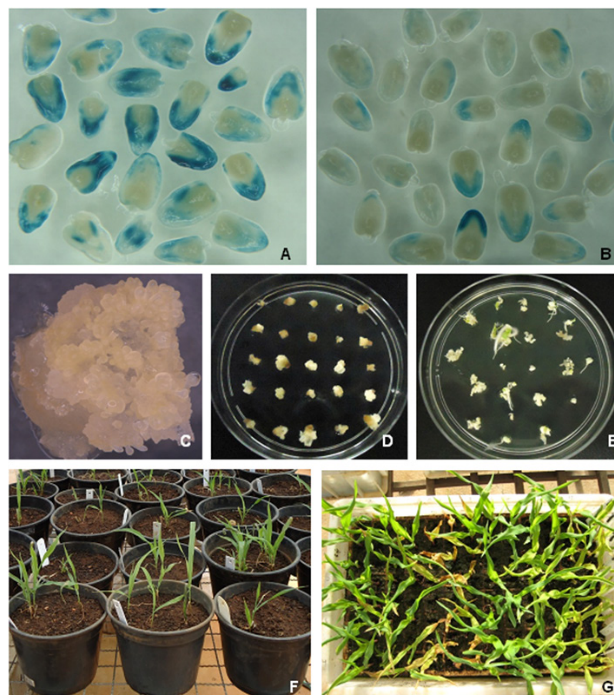

Figure 1. Genetic transformation of the L3 tropical maize inbred line. (A - B) Histochemical analysis of *uid*A gene expression; (A) infection with 50% (medium 2) and (B) 100% (medium 1) N6 salts; (C - D) Embryogenic callus after 5 weeks under bialaphos selection; (E) Selected calli on regeneration medium; (F) Plantlets in the greenhouse; and (G) T2 plants sprayed with Finale©.

calli from medium 1 and 2 matured, respectively. Mature calli were transferred to the germination medium, and at least three plants from each event were transferred and acclimatized in the greenhouse (Figure 1E and F). Plants grown in the greenhouse had normal structure, good tassel quality, and set variable numbers of seeds.

Stable integration of the transgene into the L3 maize genome was confirmed by treatment of germinated T1 and T2 seeds with the herbicide Finale© (Figure 1G). The number of herbicide resistant calli selected, the number of regenerated plants, and the efficiency of recovery of transgenic events using media 1 and 2 are shown in Table 2. Corroborating the results obtained for *GUS* expression, the total number of transgenic plants for the L3 maize line was higher when full strength N6 salt was used compared to half strength (Table 2). Half strength N6 salt may not have been effective for production of transgenic events, due to the decrease observed in the embryogenic response of the L3 embryos.

Thirty-five plants were regenerated, 26 from transformation performed using medium 1, and 9 from medium 2; PCR amplification of genomic DNA was performed in all 35 events regenerated. Amplification of the BAR gene present in the binary vector pTF102 was observed in all transgenic events and was not detected in the non-transformed plants (Figure 2A). Real time PCR (qPCR) analyses of a subset of five putative transgenic events confirmed the expression of *uid*A and BAR genes (Figure 2B). As observed for the majority of transgenic plants generated by *A. tumefaciens* mediated transformation, a low copy number of the transgenes was present in the events generated (Figure 2C).

The L3 tropical maize inbred line has excellent agronomic fitness and an efficient level of *in vitro* embryogenic capacity. In this study, it was shown that infection and cocultivation media containing full strength N6 salts were more efficient than half strength for the production of transgenic events from the L3 inbred tropical maize line. Improvement of the maturation process to achieve greater efficiency of plant production should be attempted, aiming at insertion of this maize line into programs of tropical transgenic maize production.

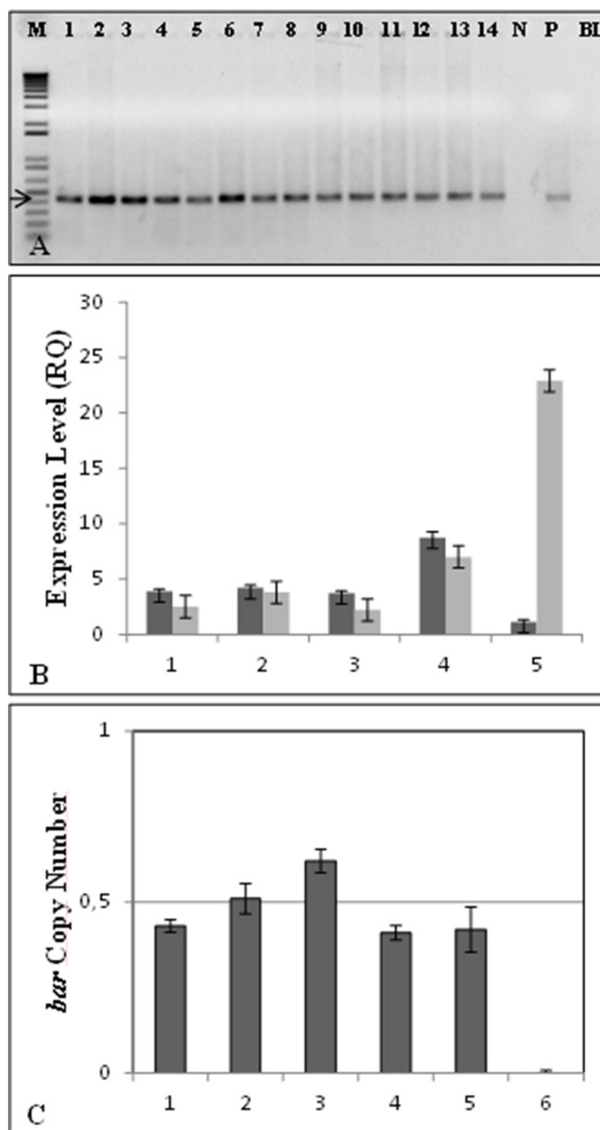

Figure 2. Molecular detection of L3 transgenic events. (A) PCR amplification of genomic DNA from 14 events; (M) molecular marker 1 kb Plus DNA Ladder (Invitrogen/SP, Brazil); (1 to 9) regenerated plants after transformation using medium 1; (10 to 14) regenerated plants after transformation using medium 2; (N) non-transgenic maize plant; (P) pTF102 binary vector; and (BL) reaction blank; (B) qPCR analysis of *uid*A (light gray bars) and BAR (dark gray bars) gene expression; (C) qPCR analysis of BAR copy number. (1, 2, and 3) events generated using medium 1; (4 and 5) events generated using medium 2; and (6) non-transgenic L3 plant; arrow corresponds to 407 bp.

ACKNOWLEDGMENTS

Our thanks to the Research Support Foundation of the State of Minas Gerais (Fundação de Amparo a Pesquisa do Estado de Minas Gerais - Fapemig) for financial support; to the Office of Improvement for Personnel in Higher Education (Coordenação de Aperfeiçoamento Pessoal de Nível Superior/Capes) for a scholarship to RAVS.

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