J. Plant Biotechnology(2000) *Vol. 2(1). pp.43~49*

Journal of Plant Biotechnology

Establishment of an Efficient *Agrobacterium*-mediated Transformation System for Eggplant and Study of a Potential Biotechnologically Useful Promoter

Claudiu Magioli[1], Ana Paula Machado da Rocha[2], Marcia Margis-Pinheiro[1], Gilberto Sachetto-Martins[1], Elisabeth Mansur[2*]

[1]*Universidade Federal do Rio de Janeiro, Instituto de Biologia, LGMV/DG - Rio de Janeiro, RJ-Brazil;* [2]*Universidade do Estado do Rio de Janeiro, Instituto de Biologia, LABMIT/DBCG-Rio de Janeiro, RJ- Brazil*

Key words: *Solanum melongena*, TDZ, tissue-specific promotor

Abstract

An efficient and reliable *Agrobacterium* transformation procedure based on TDZ (thidiazuron)-induced organogenesis was established and applied to six Brazilian eggplant varieties. Optimum transgenic plants recovery was achieved upon the study of the following parameters affecting transformation efficiency, using F-100 variety as a model: i) explant source; ii) pre-culture period; iii) physical state of the pre-culture medium and iv) co-culture conditions. The highest frequency of kanamycin-resistant calli derived from leaf explants (5%) was obtained without a pre-culture period and co-cultivation for 24 h in liquid medium followed by five days on solid RM (regeneration medium). For cotyledon explants, best results were achieved upon a pre-culture of 24 h in liquid RM and a co-cultivation period of 24 h in liquid RM followed by three days in solid RM, resulting in a transformation frequency of 22.7%. Kanamycin-resistant organogenic calli were also obtained from cultivars Emb, Preta Comprida, Round Rose Shaded, Campineira and Florida Market.

The expression pattern of an epidermis-specific promoter was studied using transformants expressing a chimaeric construct comprised by the promotor *Atgrp-5* transcriptionally fused to the coding region of the *gus* gene. The expression pattern was similar to that previously observed in tobacco and *Arabidopsis thaliana*, with preferential expression at the epidermis and the stem phloem. These results support the idea that the *Atgrp-5* promoter can be used to drive defense genes in these tissues, which are sites of pathogen interaction and spread, in programs for the genetic improvement of eggplant.

Introduction

Some protocols for stable eggplant transformation through *Agrobacterium* with both disarmed and wild strains have been published during the last decade and in recent years there have been reports on the obtention of transgenic plants harboring insect resistance genes (Chen et al., 1995; Iannacone et al., 1997; Arpaia et al., 1997). Nevertheless, eggplant transformation has generally been described as a slow and little efficient process (Rotino and Gleddie, 1990; Iannacone et al., 1997) and alternative procedures have been pursued (Chen et al., 1995). Despite the low transformation efficiencies generally achieved, no systematic comparative studies on the effect of different factors have been performed (Filipone and Lurquin, 1988; Fri et al., 1995).

One of the factors widely recognized as influencing the recovery of transgenic plants is the regeneration system used (Hiei et al., 1994). This work was designed to establish a transformation protocol using the regeneration system based on TDZ (thidiazuron)-induced organogenesis that we reported recently (Magioli et al., 1998). We analyzed the effect of: i) explant source; ii) pre-culture period;

* Corresponding author, E-mail; mansur@uerj.br.
Received Nov. 1, 1999; accepted Dec. 10, 1999

iii) physical state of the pre-culture medium and iv) co-culture conditions, using the F-100 variety as a model. We also examined the transformation competence of five other eggplant genotypes.

The transformation experiments were carried out using an *Agrobacterium tumefaciens* strain harboring a *GUS* gene fusion with the promotor of a glycine-rich protein (GRP) gene, *Atgrp-5* (de Oliveira et al., 1990; Sachetto-Martins et al., 1995). GRP's constitute a complex class of proteins which may perform distinct functions in plant cells. Although GRP's have been isolated from different plant species, little is known about their function and role in the processes of plant morphogenesis and development (Keller, 1993; Showalter, 1993). *Atgrp-5* has been shown to be preferentially expressed in protoderm-derived cells from *Arabidopsis* and tobacco (Sachetto-Martins et al., 1995). The characterization of tissue-specific promoters has received great attention from a biotechnological point of view, since they open the possibility to express genes in a regulated manner. Epidermis active promoters can be used to express defense genes at the initial site of plant-pathogen interaction. This approach has been used to express the TMV coat protein gene in tobacco and proved to be effective to promote viral resistance (Reimann-Philipp and Beachy, 1993). Thus the characteristics of *Atgrp-5* promoter make it an attractive alternative to express defense genes in the epidermis and the phloem, sites of pathogen interaction and spread, respectively.

Materials and Methods

Plant materials and culture conditions

Seeds of eggplant varieties F-100 and Emb were obtained from Agroceres Ltda.; cultivars Florida Market and Campineira were provided by CNPH (Centro Nacional de Pesquisa de Hortalias, Brazil) and Preta Comprida and Round Shaded by CATI (Coordenadoria de Assistncia Tcnica Integral, Brazil). Seeds were washed in distilled water containing 0. 02% Tween 80, surface-sterilized for 25 minutes in a 5% sodium hypochlorite solution and rinsed three times in sterile distilled water. Seeds were germinated on half-strength MS medium (Murashige and Skoog, 1962) supplemented with 1.5 % (w/v) sucrose and 0.7% agar (SIGMA). The regeneration medium (RM) used was comprised by MS supplemented with 200 nM TDZ and 3% (w/v) sucrose (Magioli et al., 1998). Rooting of shoots was induced in half-strength MS plus 1.5% (w/v) sucrose and 0. 7% agar supplemented with 11,4M IAA. Transgenic plants were micropropagated by inoculating nodal segments on half-strength MS supplemented with 1. 5% sucrose, 0.7% agar and 11,4 M IAA.

Media pH was adjusted to 5.8 with 1 N NaOH prior to autoclaving (120℃ for 15 minutes) and after addition of TDZ. Antibiotics and IAA were filter sterilized before adding to the autoclaved culture medium. Seeds or explants were cultured on 25 mL of medium in 250 mL flasks (10 seeds or 5 explants per flask). Cultures were maintained in a growth chamber at 28±2℃ under a 16 h photoperiod regime provided by cool-white fluorescent lamps (G&E) with a photon fluency of 36 $\mu M/m^2 sec$.

Bacterial strain and plasmid

Transformation experiments were performed using *Agrobacterium tumefaciens* strain C58C1 (pMP90) harboring the binary vector pTSM-3.1800 (Sachetto-Martins et al., 1995), which contains *Atgrp-5* promoter transcriptionally fused to gus reporter gene. This plasmid also contains the neomycin phosphotransferase (*npt*II) gene under the control of nopaline synthase (*nos*) promoter as selectable marker.

Agrobacterium was grown in YEB medium (10 g/L bacto peptone, 5 g/L NaCl and 10 g/L yeast extract) supplemented with the appropriate antibiotics (100 mg/L rifampicin, 40 mg/L gentamycin, 300 mg/L streptomycin and 100 mg/L spectinomycin) for 16 h at 28℃, under constant agitation.

Transformation conditions

For co-cultivation a fresh overnight *Agrobacterium* culture was diluted to about 10^7 bacteria/mL (OD_{600}= 0.01). To determine the effect of different pre-culture periods on transformation frequency, leaf and cotyledon segments ($50mm^2$) of F-100 variety were excised from 21-day-old seedlings and cultured for 0, 24, 48 and 72 h on solid or liquid RM. In the first co-cultivation step, explants were maintained in 5 mL liquid RM plus 1 mL of the diluted bacterial suspension for 24 h in the dark. In a second step, inoculated explants were blotted dry on sterile filter paper and incubated for 0, 1, 2, 3, 4 or 5 days on solid RM. After these procedures, explants were transferred to selection medium (RM supplemented by 50 mg/L kanamycin and 500 mg/L cefotaxime). Resistant organogenic calli were transferred to basal MS plus 50 mg/L kanamycin and 250 mg/L cefotaxime for bud development. Shoots were transferred to root induction medium without selection. Whole plantlets were transferred to greenhouse after an acclimatization period of 2 weeks in a phytotron chamber.

Transformation efficiency was calculated as the

percentage of explants that developed kanamycin resistant calli and of calli that originated *GUS* positive plants. Fifty explants were analyzed in each experiment and each treatment was repeated at least three times. Intervals of confidence were given for = 0.05 using the t-test.

Histochemical GUS detection

Histochemical GUS assays were carried out according to Jefferson et al. (1987) with minor modifications. Plant organ sections were cut with a Vibratome (Campden Instruments Ltd, Belgium) and submersed in staining solution containing 2mM of 5-bromo-4-cloro-3-indolyl-D-glucuronide (X-gluc) in 100 mM phosphate buffer, pH 7, in the presence of 2 mM ferricyanide and 0.5mM ferrocyanide. Samples were incubated at 37°C for 16 h. Green tissues were incubated in ethanol after the assay in order to remove the chlorophyll and observed under a light microscope.

Results and Discussion

Preliminary experiments performed with cotyledons from the F-100 variety showed that explants co-cultivated for 24-48 h in liquid (Fri et al., 1995) or on solid regeneration medium (Rotino and Gleddie, 1990; Arpaia et al., 1997; Iannacone et al., 1997) did not originate any kanamycin resistant calli. Therefore, we tested some other co-cultivation protocols and only obtained resistant calli and shoots from explants which were co-cultivated for 24 h in liquid RM and then placed on solid non-selective medium for three days. Thus, to study other parameters influencing transformation efficiency, we examined the influence of both pre-culture periods and explant type using this two-step (liquid + solid medium) co-cultivation procedure.

The recovery of resistant calli and shoots was greatly influenced by explant source and pre-culture periods. In cotyledon explants highest rates of kanamycin resistant calli formation were observed after pre-culture periods of 48 and 72 h in solid medium or 24 h in liquid medium (Table 1).

However, the highest frequency of *GUS*-positive shoots (22.7%) was obtained from explants which were pre-cultured in liquid medium for 24 h.

Pre-conditioning of explants before co-cultivation with *Agrobacterium* by culture in regeneration inducing medium appears to be critical for the obtention of transgenic plants from some species, such as *Arabidopsis* and flax (Sangwan et al., 1991; McHughen et al., 1989). This has been correlated to improved integration of T-DNA as a consequence of intense cell division at the injured regions of explants (McHughen et al., 1989; Muthukumar et al., 1996). In eggplant, the use of a pre-culture period of 48 h on solid medium has been described both for leaf and cotyledon explants (Rotino and Gleddie, 1990; Arpaia et al., 1990; Iannacone et al., 1997). Fri et al. (1995) reported that pre-conditioning on liquid medium resulted in a two-fold increased transformation efficiency relative to control explants. In the system described here, we demonstrated that specific pre-culture conditions were required according to ex-

Table 1. Effect of pre-culture in the frequency of transformation.

Pre-culture period (h)	Kanamycin resistant calli (%)		Calli originating GUS+ shoot (%)	
	Leaf	Cotyledon	Leaf	Cotyledon
0	67.5	43.3	0	8.3
		Solid RM medium		
24	3.3	48.7	0	10
48	58.6	67.5	0	5
72	31.5	63.8	0	0
		Liquid RM medium		
24	5	70	0	22.7
48	60	43.9	0	0
72	33.3	32.5	0	0

Leaf and cotyledon explants were pre-cultivated in solid or liquid RM, co-cultivated with *Agrobacterium* for 24 h and maintained in solid MR medium for 3 days before transfer to selective medium.

plant type.

Leaf explants either without a pre-culture period or after 48 h on both solid and liquid medium displayed high rates of callus formation in selective medium. However, no *GUS*-positive shoots were obtained from these calli, using the co-cultivation schedule adopted for the evaluation of the effect of different pre-culture periods (1 d in liquid followed by 3 d on solid RM medium). Hence, we also evaluated the effect of different periods for the second co-cultivation step both for leaf and cotyledon explants. While cotyledon explants showed highest rates of callus production (approximately 70%) after a second step period of 2-4 days, the highest rate of callus formation (86%) from leaf explants was obtained with a second step period of 4 d (Figure 1).

Although leaf and cotyledon explants exhibited similar rates of callus formation, the development of shoots from the resistant calli was markedly different in each and was also influenced by the co-cultivation schedule adopted. Shoot development was obtained from calli derived from leaf explants only when a second co-cultivation step of 4-5 days was adopted. On the other hand, *GUS*-positive shoots were obtained from 22.7% of cotyledon-derived calli, upon the use of a second co-cultivation step of 3 days (Figure 1).

In agreement with other reports, co-culture conditions were an important factor for the success of the transformation system described here. The critical influence of this parameter has been attributed to a number of reasons. Short co-culture periods may not be sufficient for *Agrobacterium* infection and long periods may either allow excessive bacterial growth or affect the regeneration competence of the explant (Fillatti et al., 1987). In addition, long co-culture periods also retard the introduction of selective pressure, allowing the multiplication of transformed cells and hence increasing the possibility of transgenic plants recovery. In our two-step co-cultivation procedure, the first 24 h in liquid medium may be regarded as an efficient infection procedure. The additional periods have at least three beneficial effects for the recovery of transgenic shoots: i) increased infection period; ii) culture of explants on regeneration inducing medium during infection and iii) late selection.

In order to use the optimized transformation system described above in other eggplant varieties cultured in Brazil, we first evaluated the morphogenetic capacity of each in response to 200 nM TDZ and then assessed the frequency of kanamycin-resistant calli from cotyledon explants inoculated with *Agrobacterium*. Explants from all cultivars showed high frequencies of organogenic callus induction. Among

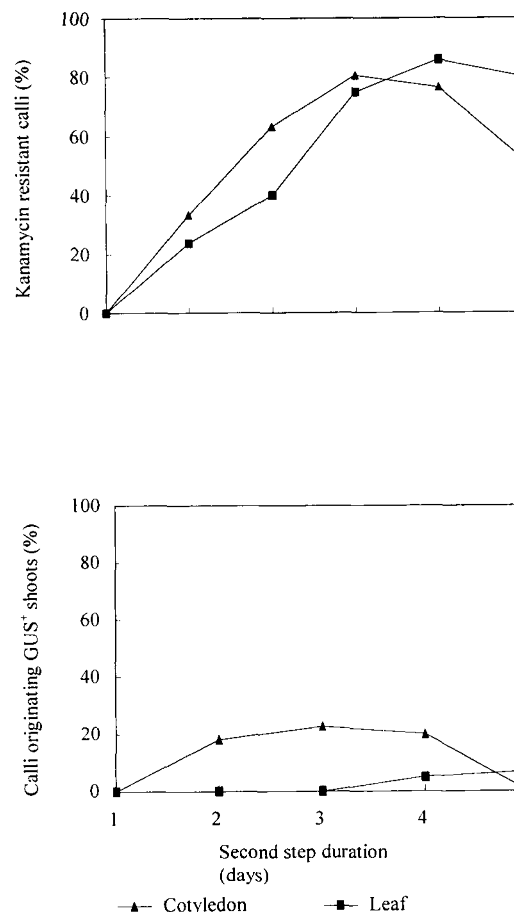

Figure 1. Effect of different second step periods of co-cultivation on solid MR medium following the first co-cultivation step (24h in liquid MR medium). Leaf explants were co-cultivated immediately after excision and cotyledon explants were pre-cultured for 24 h in liquid RM.

them, Emb, Round Rose Shaded and Preta Comprida showed the highest numbers of bud/explant as well as the highest frequencies of kanamycin-resistant calli formation (Table 2).

Transgenic plants obtained were phenotypically normal and fertile.

Taken together, these results demonstrate that the regeneration system based on TDZ is suitable for eggplant transformation mediated by *Agrobacterium*. The protocol described here (summarized in Figure 2) resulted in transformation efficiencies between 15 and 55% in five different genotypes, whereas protocols described previously for other varieties resulted in rates around 7% (Guri and Sink, 1988; Filip-

pone and Lurquin, 1989; Rotino and Gleddie, 1990; Fri et al., 1995). Although transgenic plants recovery was obtained from both leaf and cotyledon explants, transformation rates observed in cotyledons were significantly higher. This was an expected result, considering that our previous studies have shown that cotyledons are more competent for TDZ-induced organo genesis (Magioli et al., 1998) and also the most responsive to infection by wild strains of *Agrobacterium* (Magioli, 1995).

The analysis of *Atgrp-5* promoter activity in eggplant showed that its qualitative expression pattern was identical for the different transgenic lines analyzed. However, some variation in the relative levels of GUS expression was detected, as is commonly observed in *gus*-promoter fusion experiments. These variations are usually correlated with positional effects from insertion of the gene construct in different sites of the genome. To obviate concern about the influence of position effects on the pattern of expression, all observations were made on 7 eggplant transgenic lines.

In leaves, GUS expression was restricted to the epidermal layer including the trichomes (Figure 3A) and guard cells (Figure 3B). Similar results were obtained in petioles (data not shown). In stem, *Atgrp-5* promoter activity was detected in the epidermis (Figure 3C) and phloem (Figure 3C). In roots, expression was observed in the root apex (Figure 3D), vascular tissue and during initiation of lateral roots (Figure 3E).

Atgrp-5 promoter was also active in reproductive organs of eggplant. In anthers, GUS expression was detected in epidermal cells from the anther filament (data not shown) and stomium (Figure 3F). The promoter was also active in the stigma (Figure 3G). In the ovary of open flowers, GUS expression was ob-

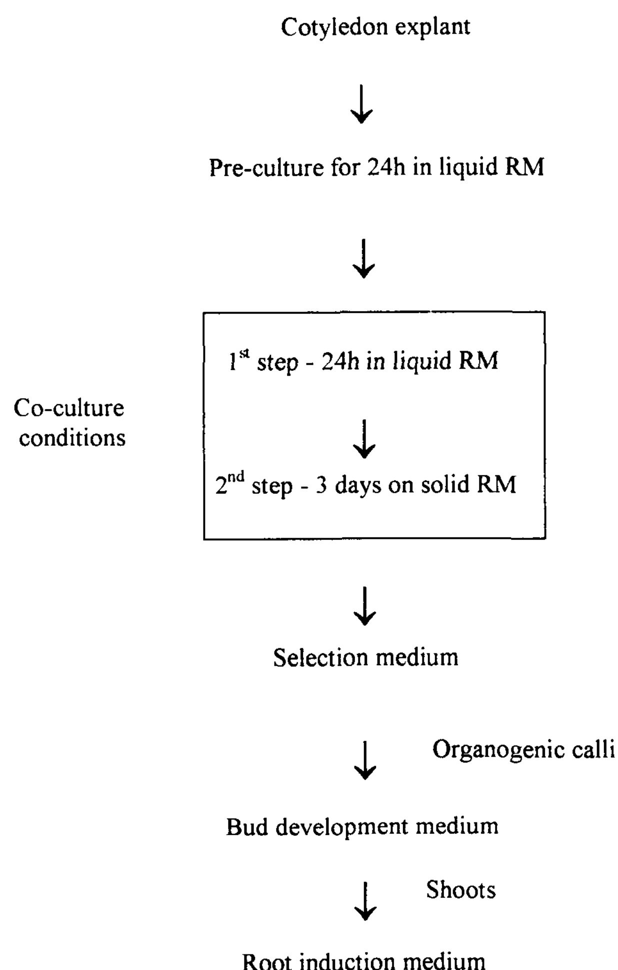

Figure 2. Schematic representation of the transformation protocol.

Table 2. Organogenic response to 200 nM TDZ and transformation competence of different eggplant genotypes.

Genotype	Organogenic response		Transformation response
	Callus induction (%)	Mean number of buds/explant (± SE)	Kanamycin resistant calli (%)
Embú	91	40 (3.6)	55
Campineira	Nd[a]	Nd[a]	15
Florida Market	100	8.4 (±2.0)	22.7
Round Rose Shaded	83.3	26.2 (±4.5)	52.3
Preta Comprida	100	28.4 (±2.4)	30

[a]Nd Not determined
Cotyledon explants were pre-conditioned for 24 h in liquid RM, co-cultivated in liquid RM and maintained on RM for 3 days before transfer to selective medium. Callus formation was assessed after 30 days of culture.

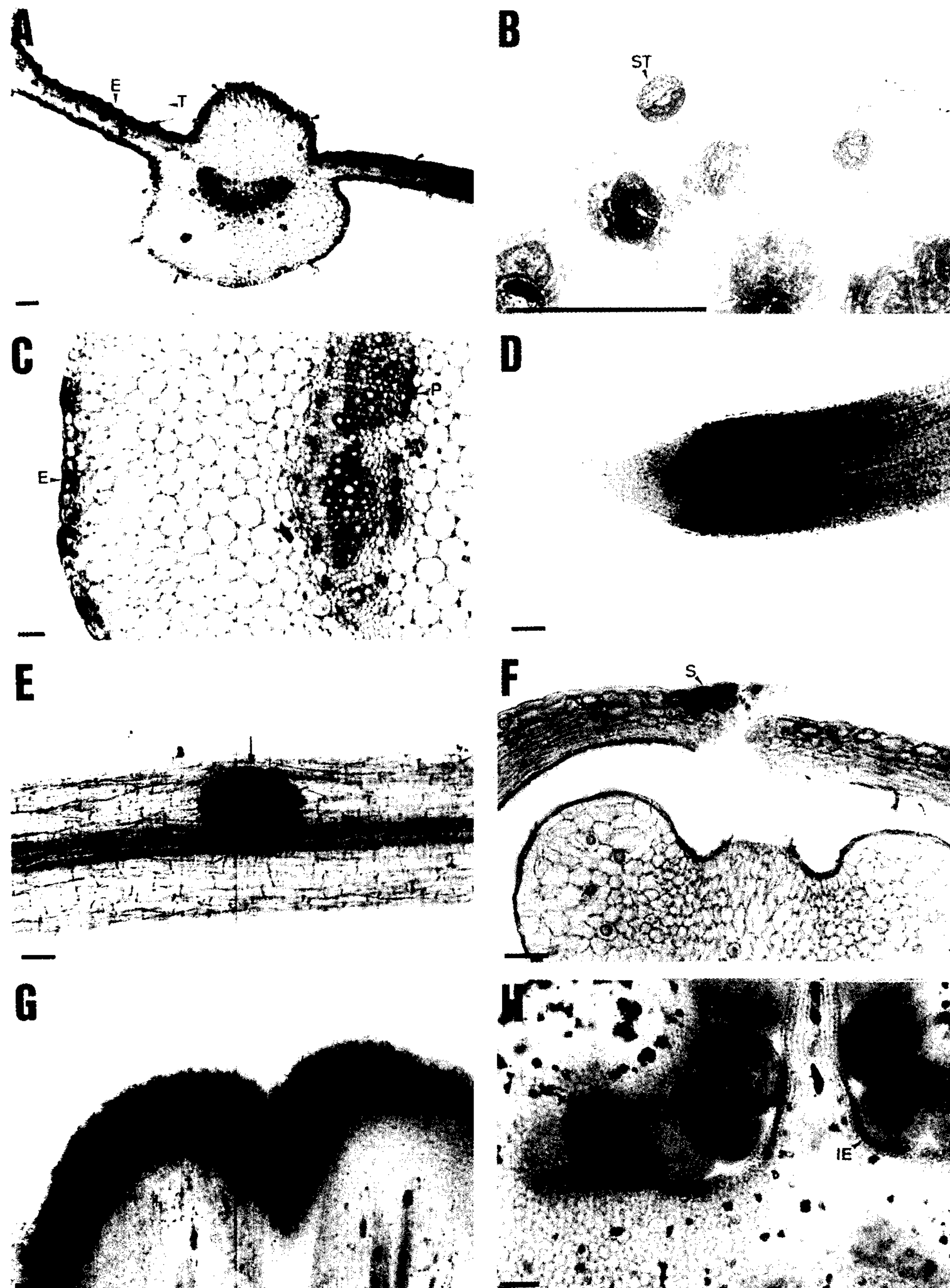

Figure 3. Activity of *Atgrp-5* promoter in eggplant. Histochemical localization of GUS expression in transgenic plants. A - Cross section of leaf. B - Frontal view of leaf epidermis. C - Cross section of stem. D - Root tip. E - Lateral root initiation. F - Cross section of anther. G - Longitudinal section of stigma. H - Cross section of ovary. E = epidermis, T = trichome, ST = stomata, P = phloem, S = stomium, IE = inner epidermis. Bar = 10M

served at the inner epiderm and developing ovules (Figure 3H). Hence, the activity of *Atgrp-5* promoter in eggplant was quite similar to that observed in *Arabidopsis* and tobacco transgenic plants (Sachetto-Martins et al., 1995), being restricted to protoderm-derived tissues.

In conclusion, in this work we have developed an efficient and reliable protocol for six eggplant varieties commonly cultivated in Brazil, including Florida Market and Campineira which are used in hybrid production. In addition, we demonstrated that the expression pattern of *Atgrp-5* in eggplant is similar to that observed in *Arabidopsis* and tobacco, opening the possibility to use this promotor to drive resistance genes against pathogens which attack the epidermis and/or spread via phloem.

Acknowledgments

The authors thank L.Frade, M. Sabino and F. Barbosa. for the excellent technical assistance and to Dr. M. Lima for critical reading of the manuscript. C. Magioli, A.P.M. Rocha and E. Mansur were recipients of fellowships from CNPq (Conselho Nacional de Desenvolvimento Cientfico e Tecnolgico).

References

Arpaia S, Mennella V, Onofaro V, Perri E, Sunseri F, Rotino GL (1997) Production of transgenic eggplant (*Solanum melongena* L.) resistant to Colorado Potato Beetle (*Leptinotarsa decemlineata* Say). Theor Appl Genet 95: 329-334.

Chen Q, Jelenkovic G, Chin CK, Billings S, Ederhardt J (1995) Transfer and transcriptional expression of coleopteran *cry*IIIB endotoxin gene of *Bacillus thuringiensis* in eggplant. J Amer Soc Hort Sci 120: 921-927.

de Oliveira DE, Seurinck J, Inz D, Van Montagu M, Botterman J (1990) Differential expression of five *Arabidopsis* genes encoding glycine-rich proteins. Plant Cell 2: 427-436.

Fri M, Nagy I, Csnyi M, Mityk J, Andrsfalvy A (1995) *Agrobacterium* mediated genetic transformation and plant regeneration via organogenesis and somatic embryogenesis from cotyledon leaves in eggplant (*Solanum melongena* L. cv. "Kecskemti lila"). Plant Cell Rep 15: 82-86.

Fillatti JJ, Sellmer J, McCown B, Haissing B, Comai L (1987) *Agrobacterium*-mediated transformation and regeneration of hybrid aspen and poplar clones. Plant Physiol 93: 1110-1116.

Fillipone E, Lurquin PF (1989) Stable transformation of eggplant (*Solanum melongena* L.) by cocultivation of tissues with *Agrobacterium tumefaciens* carrying a binary plasmid vector. Plant Cell Rep 8: 370-373.

Guri A, Sink KC (1988) *Agrobacterium* transformation of eggplant. J Plant Physiol 133: 52-55.

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6: 271-282.

Iannacone R, Grieco PD, Cellini F (1997) Specific sequence modifications of a *cry*3B endotoxin gene result in high levels of expression and insect resistance. Plant Mol Biol 34: 485-496.

Jefferson RA, Kavanagh A, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. Embo J 6: 3901-3907.

Keller B (1993) Structural cell wall proteins. Plant Physiol 101: 1127-1130.

Magioli C (1995) Establishment of regeneration and transformation systems via *Agrobacterium tumefaciens* for eggplant (*Solanum melongena* L.). M.Sc. Thesis, Universidade Federal do Rio de Janeiro, Brazil.

Magioli C, Rocha APM, de Oliveira DE, Mansur E (1998) Efficient shoot organogenesis of eggplant (*Solanum melongena* L.) induced by thidiazuron. Plant Cell Rep 17: 661-663.

McHughen A, Jordan M, Feist G (1989) A preculture period prior to *Agrobacterium* inoculation increase production of transgenic plants. J Plant Physiol 135: 245-248.

Murashige R, Skoog F (1962) A revised method for rapid growth and bioassays with tissue cultures. Plant Physiol 15: 473-497.

Muthukumar B, Mariamma M, Velulhambi K, Gnanam A (1996) Genetic transformation of cotyledon explants of cowpea (*Vigna unguiculata* L. Walp) using *Agrobacterium tumefaciens*. Plant Cell Rep 15: 980-985.

Reimann-Philipp U, Beachy RN (1993) Coat protein-mediated resistance in transgenic tobacco expressing the tobacco mosaic virus coat protein from tissue-specific promoters. Mol Plant Micr Int 6: 323-330.

Rotino GL, Gleddie S (1990) Transformation of eggplant (*Solanum melongena* L.) using a binary *Agrobacterium tumefaciens* vectors. Plant Cell Rep 9: 26-29.

Sachetto-Martins G, Fernandes LD, Felix DB, de Oliveira DE (1995) Preferencial transcriptional activity of a glycine-rich protein gene from *Arabdopsis thaliana* in protoderm-derived cells. Int J Plant Sci 156: 460-470.

Sangwan RS, Bourgeois Y, Sangwan-Norrel BS (1991) Genetic transform ation of *Arabidopsis thaliana* zygotic embryos and identification of critical parameters influencing transformation efficiency. Mol Gen Genet 230: 475-485.

Showalter AM (1993) Structure and function of plant cell wall proteins. Plant Cell 5: 9-23.