J. Appl. Genet. 42(4), 2001, pp. 405-412

Particle-inflow-gun-mediated genetic transformation of buffel grass (*Cenchrus ciliaris* L.): optimizing biological and physical parameters

Vishnu BHAT[1], Sue J. DALTON[2], Suresh KUMAR[1], Basrur Venkatesh BHAT[1], Madan G. GUPTA[1], Phillip MORRIS[2]

[1] Biotechnology Section, Division of Crop Improvement, Indian Grassland & Fodder Research Institute, Jhansi, India
[2] Institute of Grassland & Environmental Research, Aberystwyth, U.K.

Abstract. The present study was conducted to optimize various biological and physical parameters for developing an efficient and reproducible gene transfer method for genetic transformation of buffel grass. Transformation was carried out using a helium-driven particle inflow gun (PIG). Embryogenic calli produced from mature seeds of buffel grass cv. CC-119 were separately bombarded with four plasmids, containing Actin (pAct1DX), Ubiquitin (pAHC-25; pAHC-27) and CaMV-35S (pCaMVGUS) promoters, coated on tungsten and gold particles. The efficiency of transformation was monitored through transient GUS expression. Different parameters, *viz.*, the type of promoter, type and size of microcarrier, helium gas pressure, distance and time of bombardment, were standardized for delivering DNA into embryogenic calli. Bombardment with plasmid DNA carrying the actin promoter coated on 1.6 μ gold particles, at a helium pressure of 4 bars, a distance of 10 cm for 10 μ sec and 28 mm Hg vacuum in the chamber, produced the best result in transient GUS expression. The Actin promoter was found to be more efficient in driving expression of the GUS gene in buffel grass, followed by Ubiquitin and CaMV-35S promoters. Lower helium pressure was found to be sub-optimal, while higher pressure produced a smaller number of blue spots, probably due to excessive damage to the cells. Maximum of 385 blue spots was observed with gold particles of 1.6 μ size, whereas only 213 blue spots were recorded for tungsten particles of 1.0 μ size. The optimized parameters can be employed for genetic transformation of buffel grass with genes of agronomic importance.

Key words: buffel grass, *Cenchrus ciliaris*, genetic transformation, particle inflow gun, promoters.

Received: March 15, 2001. Accepted: September 10, 2001.
Correspondence: V. BHAT, Biotechnology Section, Division of Crop Improvement, Indian Grassland and Fodder Research Institute, Jhansi – 284 003 (U.P.) India, e-mail: bhatv@lycos.com

Introduction

Buffel grass (*Cenchrus ciliaris* L.) is an apomictic, perennial, polymorphic and polyploid warm-season forage grass with an extensive native range from the tropics of Africa to India (HIGNIGHT et al. 1991). It is highly drought-tolerant and is well adapted to arid and semi-arid areas. It is mainly used as a pasture grass for ruminants, and may also be used for hay or silage making. It is highly palatable to all kinds of grazing animals. However, buffel grass has poor digestibility due to its high lignin content (3-5%) (MINSON, BRAY 1986). Being an apomict, its genetic improvement through conventional breeding methods is difficult, time-consuming and presently restricted to the selection of elite lines from natural variants. Hence, genetic transformation would be a powerful tool for the improvement of this grass. Moreover, apomixis may facilitate varietal improvement by genetic transformation, since no further breeding is required to fix the transferred character (VIELLE CALZADA et al. 1996). Prerequisites for successful transformation are (1) efficient techniques of plant regeneration, and (2) delivery of foreign DNA into regenerable cells. Availability of a protocol for genetic engineering of buffel grass, for example, in manipulating the lignin pathway to increase forage digestibility, would be very helpful. A preliminary study of transient expression of a reporter gene following particle bombardment has been reported in buffel grass (ROSS et al. 1995). Availability of a more efficient transformation method would facilitate the production of useful transgenic plants in superior cultivars. The efficiency of transformation methodology is dependent on genotype, *in vitro* culture conditions, and both biological and physical parameters employed for gene delivery. The objective of this work was to optimize biological and physical parameters for PIG-mediated genetic transformation of embryogenic calli of buffel grass.

Material and methods

Plant material

Mature seeds of buffel grass (*Cenchrus ciliaris* L.), genotype CC-119, were used as starting material. Seeds were dehusked, surface-sterilized in 70% ethanol for 1 minute, followed by 1.5% (v/v) sodium hypochlorite with a drop of Tween-20 for 30 minutes, and then rinsed several times with sterile distilled water. Seeds were placed on Murashige and Skoog (MS) medium (MURASHIGE, SKOOG 1962) supplemented with 22.6 μM 2,4-D, 0.84 μM BAP and incubated at 26 ± 1°C in the dark. After a week of incubation, calli induction was observed on the germinated seeds. Following three to four weeks of *in vitro* culture, calli contained both embryogenic and non-embryogenic regions. Embryogenic regions were excised and subcultured on the same callusing medium freshly prepared at an interval of three weeks for two to three passages.

Plasmid DNA

Plasmid DNA containing the *uidA* (GUS reporter) gene under the control of different promoter-intron combinations and the *nos* ending region were used. Plasmid pAHC-25 (CHRISTENSEN et al. 1992) contains the *uidA* reporter gene encoding β-glucuronidase enzyme and a selectable marker, the *bar* gene encoding the phosphinothricin acetyl transferase, which confers resistance to the herbicide Basta, under control of the maize ubiquitin-1 promoter-first exon-first intron and the *nos* ending region. Plasmid pAHC-27 contains the *uidA* gene under the maize ubiquitin-1 promoter-first intron and the *nos* ending region (CHRISTENSEN, QUAIL 1996). Plasmid pAct1DX contains the *uidA* gene under control of the rice actin-1D promoter-first intron and the *nos* ending region (MCELROY et al. 1990). Plasmid pCaMVGUS contains the *uidA* reporter gene driven by the CaMV-35S promoter.

Particle bombardment

Transformation experiments were carried out using a helium-driven particle inflow gun constructed according to the descriptions of FINER et al. (1992), with plasmid DNA-coated microcarriers using the method described by SPANGENBERG et al. (1995). Two types of microcarriers (tungsten and gold particles) were used for the delivering of the plasmidic constructions. Microcarriers were driven by helium gas at variable pressures (Table 1). The microprojectiles were sterilized by vortexing 5 µg of particles with 100 µl of absolute ethanol in an Eppendorf tube for 1-2 minutes, followed by pelleting down and washing the particles with sterile water three times. Finally the particles were resuspended in 50 µl of sterile water. Ten microliters of suspension of surface-sterilized particles were transferred to an Eppendorf tube along with 5 µl of plasmid DNA (1 µg/µl DNA), 20 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine. The content was thoroughly mixed and incubated in ice for 10 minutes. Then the mixture was centrifuged and resuspended in 20 µl of sterile water. Ten microliters of the mixture was used for each bombardment. The target tissues (embryogenic calli) were arranged on osmotic medium (MS medium with 0.2 M sorbitol and 0.2 M mannitol) in the centre of 90-mm Petri plates on a 5.5-cm filter paper disk an hour before bombardment. Pretreatment of calli on a high osmotic medium is known to increase the efficiency of transformation (VAIN et al. 1993a). The physical parameters employed for the bombardment of the calli are summarized in Table 1. After bombardment, calli were incubated on the same osmotic medium for another 48 h in the dark at 26 ± 1°C.

GUS histochemical assay

The transient expression of the *uidA* (GUS) gene was determined by a standard histochemical assay (JEFFERSON 1987) in randomly selected calli, 48 h after bom-

Table 1. Parameters used for optimizing the genetic transformation of buffel grass

Parameters	Description
Distance of bombardment	5, 10 and 15 cm
Microcarriers	tungsten and gold particles
Size of microcarriers	0.6 and 1.6 μ (gold); 1.0 μ (tungsten)
Culture medium	MS medium supplemented with 2,4-D (22.6 μM), sorbitol (0.2 M) and mannitol (0.2 M)
Plant materials	embryogenic calli arranged in centre of 90 mm Petri plate
Plasmid DNA (promoters)	pCaMVGUS (CaMV-35S), pAHC-25, pAHC-27 (Ubiquitin) and pAct1DX (Actin)
Helium pressure	2, 4, 5 and 6 bars
Vacuum	28 mm Hg
Bombardment time	1, 10 and 50 μ sec
Post-bombardment treatment of calli	incubation on osmotic medium for 48 h, followed by callusing medium till GUS assay

bardment. The embryogenic calli, spread over each filter paper disk, were divided into four quarters, 25% of the calli were selected randomly from each quarter and used for the GUS assay. Selected tissue was immersed in a sufficient volume of GUS assay solution and incubated at 37°C overnight. The number of blue spots on assayed calli was counted under the microscope and the total number was calculated as four times the observed number for each plate. The experiments were replicated three times. The total number of blue spots per Petri plate was analysed using the analysis of variance. Duncan's multiple range test was used to find significant differences among treatment means at 5% level of significance.

Results and discussion

Various parameters (Table 1) were analysed for optimizing the genetic transformation of buffel grass calli stimulated by an inflow gun device. The lower helium pressure (2 bars) was found to be sub-optimal, while the pressure of 4 bars was found to be the best assayed for GUS expression (Table 2; Figure 1). Higher pressure (5-6 bars) produced a smaller number of blue spots, probably due to excessive damage to the cells. Therefore, further optimizations were carried out using 4 bars of helium pressure. GUS gene expression was not detected in control, non-bombarded calli.

Subsequently, bombardment time and distance of bombardment were assayed (Table 2). The maximum number of blue spots was observed when calli were

Table 2. Effect of various physical parameters on the delivery of foreign DNA (pAct1DX) in terms of the *uidA* gene expression in the bombarded calli

Constants	50 μ sec, 10 cm				4 bars, 10 cm			10 μ sec, 4 bars		
Variables	Pressure (bars)				Time (μ sec)			Distance (cm)		
	2	4	5	6	1	10	50	5	10	15
No. of GUS foci[φ]	10^{a}	321^{d}	208^{c}	168^{b}	125^{a}	525^{c}	155^{b}	190^{a}	325^{c}	215^{b}

[φ] Each value is the mean of three replications.
[abcd] Entries followed by different letters are significantly different at 5% level of Duncan's multiple range test.

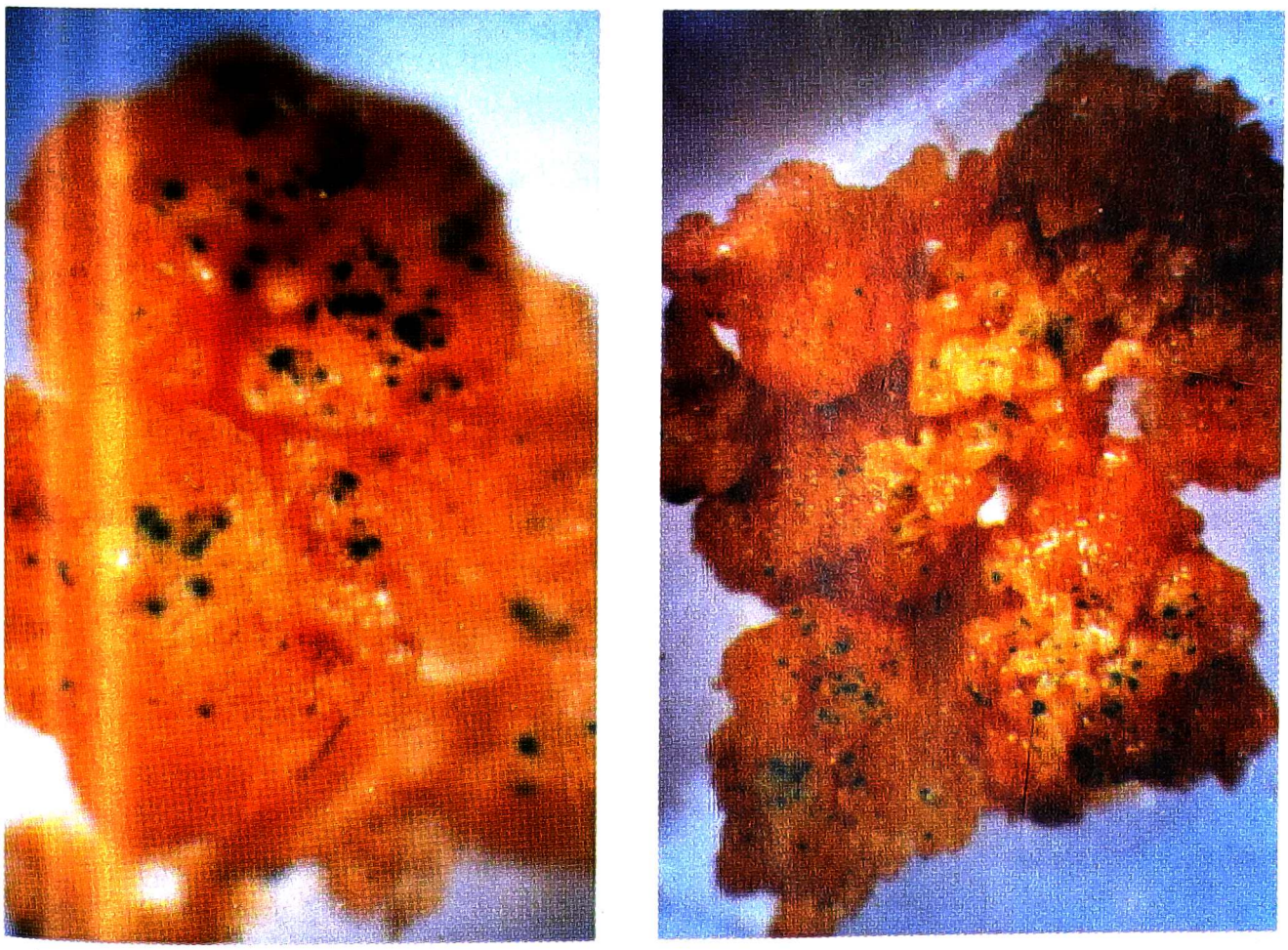

Figure 1. GUS expression on calli of buffel grass bombarded with pAct1DX

bombarded for 10 μ sec at a distance of 10 cm from the nozzle (at 4 bars of helium pressure). When tungsten and gold particles of variable sizes were used as microcarriers of pAct1DX, a maximum of 385 blue spots was observed with gold particles of 1.6 μ size, whereas only 213 blue spots were recorded for tungsten particles of 1.0 μ size (Figure 2). Similar results were obtained by VAIN et al. (1993b) in maize-suspension-cultured cells.

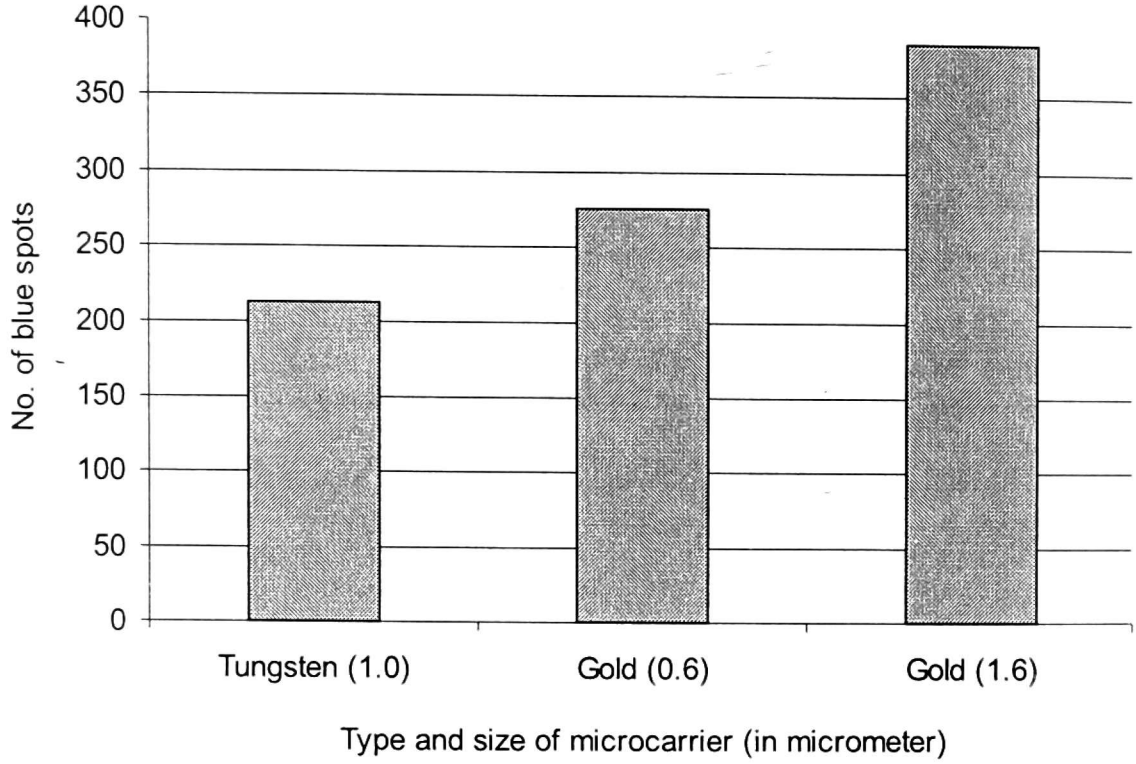

Figure 2. Effect of type and size of microcarrier used for delivery of foreign DNA (pAct1DX) in terms of frequency of the *uidA* gene expression in bombarded calli of buffel grass

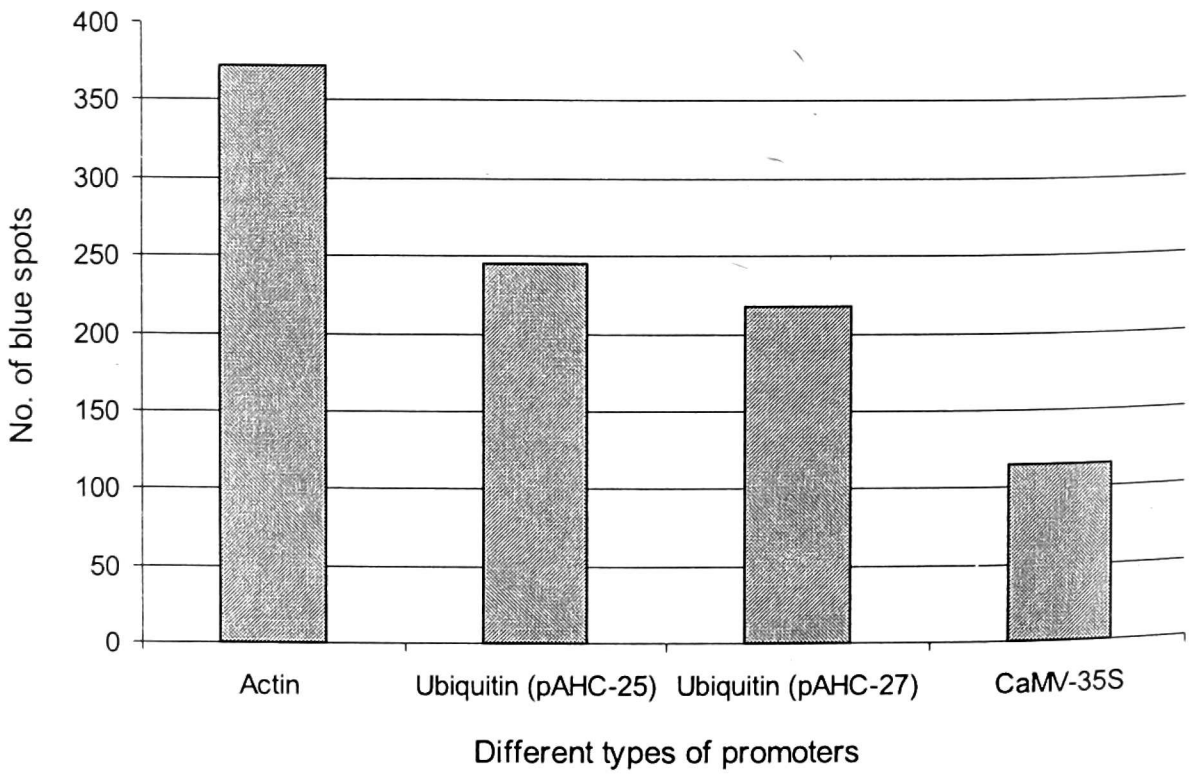

Figure 3. Effect of different promoters, driving the reporter gene in plasmid constructs, on the *uidA* gene expression in bombarded calli of buffel grass

Transformation efficiency is deeply affected by the strength of the promoter driving the introduced gene (ORTIZ et al. 1997). Actin, a promoter from monocotyledonous species, was found to be more efficient in driving expression of the GUS gene in buffel grass, followed by the Ubiquitin promoter (another pro-

moter from monocotyledonous species) and the CaMV-35S promoter from dicotyledonous species (Figure 3). In general, promoters isolated from monocotyledons show a higher activity in monocotyledonous species (WILMINK et al. 1995). Under optimum conditions maximum blue spots (525) were observed with plasmid pAct1DX (Table 2). ROSS et al. (1995) observed that by increasing the distance, transient expression increased to about 200 blue spots at 12 cm in embryogenic calli from mature seeds of cv. Biloela. This result was obtained using the pEmuGN plasmid for bombardment, where the GUS gene is driven by an Emu promoter. They also reported a maximum of 425 blue spots from a single bombardment under conditions not mentioned. Information on the efficiency of different promoters would be useful in the construction of plasmid vectors for efficient expression and regulation of foreign gene(s) in the target cells. Development of a system for production of transgenic plants needs to be assisted by a higher rate of plantlet regeneration from pre-embryoids and rapid selection procedures to avoid declining regenerability during selection of transformed cells. These standardized bombardment conditions will be utilized for the development of transgenic plants harbouring genes of interest, particularly for forage quality improvement.

Acknowledgements. The authors thank Peter H. QUAIL, Alan H. CHRISTENSEN, Andy J. BETTANY and Ray WU for providing plasmid constructs, and the anonymous reviewer for constructive suggestions. A part of the work was supported by a collaborative grant from the ODA between IGER, U.K. and IGFRI, India.

REFERENCES

CHRISTENSEN A.H., QUAIL P.H. (1996). Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transg. Research 5: 213-218.

CHRISTENSEN A.H., SHARROCK R.A., QUAIL P.H. (1992). Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplast by electroporation. Plant Mol. Biol. 18: 675-689.

FINER J.J., VAIN P., JONES M.W., MCMULLEN M.D. (1992). Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Rep. 11: 323-328.

HIGNIGHT K.W., BASHAW E.C., HUSSEY M.A. (1991). Cytological and morphological diversity of native apomictic buffelgrass *P. ciliare* (L.). Bot. Gaz. 152: 214-218.

JEFFERSON R.A. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. 5: 387-405.

MCELROY D., ZHANG W., CAO J., WU R. (1990). Isolation of an efficient actin promoter for use in rice transformation. Plant Cell 2: 163-171.

MINSON D.J., BRAY R.A. (1986). Voluntary intake and in vivo digestibility by sheep of five lines of *C. ciliaris* selected on the basis of preference rating. Grass Forage Sci. 41: 47-52.

MURASHIGE T., SKOOG F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15: 473-479.

ORTIZ J.P.A., RAVIZZINI R.A., MORATA M.M., VALLEJOS R.H. (1997). A rapid system for studying foreign gene expression in wheat (*Triticum aestivum* L.). J. Appl. Genet. 38: 123-130.

ROSS A.H., MANNERS J.M., BIRCH R.G. (1995). Embryogenic callus production, plant regeneration and transient gene expression following particle bombardment in the pasture grass, *C. ciliaris* (Gramineae). Aust. J. Bot. 43: 193-199.

SPANGENBERG G., WANG Z.Y., WU X., NOGEL J., POTRYKUS I. (1995). Transgenic perennial ryegrass (*Lolium perenne*) plants from microprojectile bombardment of embryogenic suspension cells. Plant Sci. 108: 209-217.

VAIN P., MCMULLEN M.D., FINER J.J. (1993a). Osmotic treatment enhances particle bombardment mediated transient and stable transformation of maize. Plant Cell Rep. 12: 84-88.

VAIN P., NOEL K., JESUS M., CARL R., CHERI N., FINER J.J. (1993b). Development of the particle inflow gun. Plant Cell Tiss. Org. Cult. 33: 237-246.

VIELLE CALZADA J.P., CRANE C.F., SELLY D.M. (1996). Apomixis: the asexual revolution. Science 274: 1322-1323.

WILMINK A., VAN DE VEN B.C.E., DONS J.J.M. (1995). Activity of constitutive promoters in various species from the Liliaceae. Plant Mol. Biol. 15: 809-819.