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Plantain (*Musa* spp. cv. 'Navolean' AAB) transgenic plants from *Agrobacterium tumefaciens*-mediated transformation of embryogenic cell suspensions

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ABSTRACT
The present work was carried out with the objective to obtain plantain transgenic plants of 'Navolean' (*Musa* AAB) cultivar and to evaluate them, under field conditions during the first crop cycle, for Black Sigatoka disease. Embryogenic cell suspensions obtained from scalp were used for the transformation by *Agrobacterium tumefaciens*. The bacterial strain EHA-105 was used with the binary plasmid pHGA-91, which contained a combination of genes that encode for the antifungal glucanase enzyme and the AP24 osmotin. Twenty putative transformed lines of the construct were obtained after two months of selection in the culture medium. The transgenic events were verified by means of PCR and Southern hybridisation. These confirmed the stable integration and expression of the transgenes in transgenic plants that were selected in field showing differences with non-transgenic plants used as control.

Key words: genetic transformation, AP24, glucanase, *Musa, Mycosphaerella fijiensis*

RESUMEN
El presente trabajo fue realizado con el objetivo de obtener plantas transgénicas de plátano cultivar 'Navolean' (*Musa* AAB) y evaluar estas bajo condiciones de campo durante el primer ciclo de cultivo para la enfermedad Sigatoka negra. Suspensiones celulares embriogénicas obtenidas desde domos meristematicos fueron usadas para la transformación por *Agrobacterium tumefaciens.* La cepa de bacteria EHA-105 fue utilizada con el plásmido binario pHGA-91, el cual contenía combinaciones de los genes que codifican para las antifúngicas enzimas glucanasa y la osmotina ap24. Veinticuatro putativas líneas transformadas fueron obtenidas después de dos meses de selección en medio de cultivo. Lo eventos transgénicos fueron verificados por la vía de PCR e hibridación *Southern* y estos confirmaron la integración estable y expresión del transgén en las plantas transgénicas que fueron seleccionadas en el campo y que mostraron diferencias con las plantas no transgénicas usadas como control.

Palabras clave: transformation genética, ap24, glucanasa, *Musa, Mycosphaerella fijiensis*

INTRODUCTION

The area of transgenic crops experienced a growth record in 2004, reaching a total of 81 million hectares. According to James (2004) the global area of transgenic crops grew by 13.3 million hectares in 2004, that is almost 20% increase with regard to 2003. The main crops were Soybean, Maize, Cotton and Canola, however, so far commercial GM crop of importance do not exist for food in developing countries.

The cultivation of bananas and plantains are broadly distributed in the tropical and subtropical regions of the world. The production is about 700 million tons per year with a calculated area planted of 10 million hectares. This crop is part of the alimentary diet for more

than 400 million people and it is in the fourth place in the category of nutritious products of great demand after rice, corn and wheat (FAO, 2003). Black leaf streak (*Mycosphaerella fijiensis* Morelet) is the most damaging disease for *Musa* plantation in the world, including Cuba. It is present in all areas of the country and it replaced Yellow Sigatoka Disease (*Mycosphaerella musicola*) (Pérez, 2003).

Chemical control and cultural practices reduce the affectation but does not solve the problem. Genetic transformation techniques represent an alternative to obtain *Musa* genotypes resistant to Black leaf streak (Swennen, 2003). Evaluation of transgenic plants in the field is an important step in the selection strategy.

Pérez (2000) reported *Agrobacterium*-mediated transformation of embryogenic cell suspensions from different banana and plantain cultivars. Another work has been reported by; Khanna *et al*. (2004) for 'Grande Naine' (AAA) and 'Lady Finger' (AAB) banana cultivars and Chong- Pérez *et al*. (2002) for plantain hybrid 'FHIA-21' (AAAB) and Ganapathi *et al*. (2001) for cv. 'Rasthali' (AAB). Arinaitwe *et al*. (2004) reported the comparative study between *Agrobacterium* and particle bombardment-mediated transformation of four banana and plantain cultivars.

Cohen (2005) points out the existence of field trials with transgenic banana and plantain plants in some countries (Philippines, Mexico and Costa Rica) although in this case the plants are reported transformed with the fungal resistance genes. Only Gómez-Lim *et al*. (2004) mentioned the evaluation of *Musa* transgenic plants in field with antifungal genes, but in banana cultivars.

The present work was carried out with the objective to obtain and to evaluate the plantain transgenic plants from 'Navolean' (AAB) cultivar with the use of genetic transformation via *Agrobacterium tumefaciens*.

MATERIALS AND METHODS

Genetic transformation

Cell suspension cultures

Embryogenic cell suspensions (ECSs) were started from somatic embryo groups. These were obtained from scalps in ZZ semisolid culture medium and maintained according to the methodology described by Cabrera (2002) and Strosse *et al.* (2003) for the plantain cultivar 'Navolean' (*Musa* AAB).

Bacterial strain, plasmids and culture conditions

The EHA-105 strain of *Agrobacterium tumefaciens* was used carrying the pDUBAR binary vector (Figure 1). This vector was acquired from Biotechnology and Genetic Engineer Centre (CIGB) in Havana, Cuba. The bacterial cultures were previously grown on LB medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} Sodium chloride) during 24 hours at 28°C and 200 rpm. Appropriate antibiotics were included in the medium: rifampicin, ampicillin and streptomycin each at 50 mg l^{-1} and spectinomycin at 300 mg l^{-1}.

The cell culture was centrifuged to 4300 rpm during 10 min and the bacterial pellet was re-suspended in induction medium (AB salts 20X, 2 mM NaH_2PO_4, 30 mM MES, 1% glucose, 200 ìM acetosyringone, pH 5.6) with the appropriate antibiotics (Pérez, 2000). The culture was incubated at 28°C and 200 rpm overnight to reach approximately an OD_{600} = 1.0.

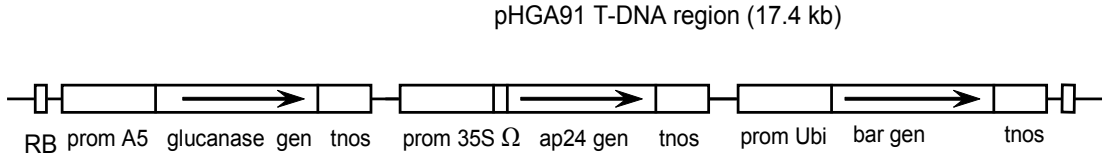

Figure 1. Schematic representation of T-DNA regions of binary vector used in genetic transformation experiments. ß-1,3-endoglucanase (PR 2) gene and AP-24 gene, osmotin protein (PR 5) from tobacco.

Inoculation and co-cultivation

The induced bacterial culture was transferred to induction medium free from antibiotics and diluted to an OD_{600} of 0.5-0.6 before infection of cells suspension. ECSs (150 ìl of cells), were used at a concentration of 33% SCV and mixed with 1 ml of the induced bacterial suspension 5 days after subculture. These were kept in darkness at 25°C and 25 rpm for 2 hours.

The infected cells suspensions were placed on nylon meshes (200 ìm) under filter paper to eliminate excessive bacteria. The meshes were transferred to semisolid ZZ multiplication medium (Strosse *et al*., 2003) supplemented with acetosyringone (200 ìM) and pH 5.6. The co-culture remainet 3 days in the dark at 21°C.

After co-culture, the cells were transferred to semisolid ZZl multiplication medium (Dhed'a *et al.*, 1991) supplemented with 200 mg l^{-1} timentine and 6 mg l^{-1} BASTA®. The cells remained in this medium for two months with subcultures every 15 days and incubated in the dark at 27°C. After this time the transformants were transferred to embryo formation medium RD1 (Dhed'a *et al.*, 1991). Embryos were formed in 1-2 months transferring then to the germination medium proposed by Kosky *et al.* (2000).

The putatively transformed *in vitro* plants were multiplied until obtaining 15-20 plants per line using M5 medium (medium proposed by Murashige and Skoog (1962) supplemented with 2.0 mg l^{-1} 6-BAP, 0.65 mg l^{-1} IAA and 30 g l^{-1} sucrose and rooted using M6 medium (Strosse *et al*., 2003): (medium proposed by Murashige and Skoog (1962) supplemented with 1.3 mg l^{-1} IAA and 40 g l^{-1} sucrose).

Acclimatization stage

Rooted plants were acclimatized according to Pérez *et al.* (1999) in a greenhouse covered with a plastic mesh, reducing light intensity to 30%. The plants were planted in Styrofoam trays of 70 orifices with a substrate composed of a mixture of casting and zeolite (3:1).

Irrigation was carried out with a microjet system, with a frequency of six irrigations per day each during 2 min. With this frequency a relative humidity of 85-90% was guaranteed.

Field evaluation

After 60 days in the acclimatization stage, 25 putative transgenic lines of the cultivar 'Navolean' (*Musa* AAB) (pHGA91) were planted in the Experimental Station in Remedios, Villa Clara, Cuba on ferralitic soil without an irrigation system. For field trials the National Biosafety Committee issued the Biological Security License.

A complete random design was used. Three replicates per line were selected, each one with three plants, using untransformed lines as controls. The parameters evaluated were done according to INIBAP Technical Guidelines 6 (Carlier *et al.*, 2002).

Evaluation

Two evaluations were done:
The first evaluation after four, five and six months of planting and the second one at shooting (bunch emergence). The following data were collected according to the criteria of Carlier *et al.* (2002):

- Youngest leaf spotted (YLS): is the leaf number of the first unfurled leaf with at least 10 discrete, mature, necrotic lesions or one large necrotic area with 10 light-colored dry centers.
- Number of functional leaves (NFL): Functional leaves are leaves that have photosynthetic activity. Considering that a leaf is functional if it has more than 50% of green area.
- Number of total leaves (NTL)
- Disease severity: leaves should be graded using Gauhl´s modification of Stover system. After an infection, index for each plant should be calculated (Only evaluated at shooting)

Molecular analysis

PCR analysis

Genomic DNA was isolated from plants of five putative transgenic lines, grown in field, with different according YLS the control untransformed. The Wizard Genomic DNA Purification Kit was used (Promega) with modifications in the extraction buffer: 100 mM Tris –HCl, pH=8; 50 mM EDTA, 500 mM NaCl, 10 mM β-ME (mercaptoethanol), 2% PVP

(polyvinyl pyrrolidone, MW 10.000), as per manufacturer's instructions. PCR with gene specific primers was used to confirm presence or absence of *bar* transgene.

The procedure followed:

PCR amplification: two micro liters of DNA preparation were used in a 20 µl reaction mixture containing buffer for Taq pol 10 X, 200 µM of each deoxynucleoside triphosphate (dATP, dTTP, dGTP and dCTP), 1µM of each primer, 1.5 mM $MgCl_2$ and 1 units Taq DNA polymerase in a 0.2 ml tube. The two deoxynucleotide primers were: *bar*F: 20 nucleotides long, *bar*R: 17 nucleotides long. The sequence of primer *bar*F was: 5' CCATCGTCAACCACTACATC 3' and *bar*R is: 5' AGAAACCCACGTCATGC 3'.

Reaction mixtures were placed in an automated thermal cycler (Mastercycler personal, Eppendorf), heated to 95 ^{0}C for 2 minutes and subjected to 35 step cycles of 30 seconds at 95^{0}C, 30 seconds at 55^{0}C, 45 seconds at 72^{0}C. The final incubation at 72^{0}C was extended for 5 minutes. The reactions were stored at -20^{0}C. A plasmid containing the pHCA58 construct was used as positive control and water control and non-transformed plants were used as negative controls.

PCR products were checked on Ethidium bromide stained 1.5 % agarose gel.

Southern blot analysis

Integration of transforming DNA from plasmid pHCA58 was analysed using Southern hybridisation to probe for presence of *bar* gene. Plasmid pHCA58 and genomic DNA from plants transformed with it were digested with *Eco*RI which cut the plasmid DNA as shown in Figure 2. Genomic digested (10 µg) and plasmid digests (5 pg) were separated by electrophoresis on a 0.8% (w/v) agarose gel run for approximately 4 hours at 40 V and transferred by capillary blotting onto positively charged nylon membrane. The HighBond N+ nylon membrane (Amersham) was wrapped in Saran wrap and baked for two hours at 80ºC. The membrane was left under vacuum overnight at room temperature (25-28ºC). The Gene Images random prime labelling kit and the Gene Images CDP-Star detection kit from Amersham were used to label a probe for the *bar* gene and detection events, respectively. A *bar* probe (20 ng/ml hybridization solution) was used for the hybridization. The membrane was hybridized overnight at 60°C according to the manual of Amersham Co. The blot was exposed overnight on X-Ray developing the film.

RESULTS AND DISCUSSION

Genetic transformation

Many cells were observed with brown to white coloration during subcultures. Cells aggregates did not grew in the control not transformed. The embryos in the globular state were visible on the surface of the embryogenic cell aggregates after four weeks of subculture in culture medium without BASTA®. Finally, the mature embryos were transferred to the germination culture medium and the coleoptiles emergency was observed after four to eight weeks.

A total of 25 putative transformed lines were obtained plasmid using (Table 1).

Table 1. Results of the genetic transformation of 'Navolean' (AAB) cultivar with the plasmid pHGA91.

Plant material transformed	Number
Total number of samples transformed.	14
Independent putative transformed colonies/per samples (%)	2.3
Total number of indep. putative transformed lines regenerated	25
Total number of indep. putative transformed lines multiplied	25

Field evaluation

First evaluation

Table 2 summarizes the results after four, five and six months after planting based on the youngest leaf spotted (YLS).

Results are average of nine plants per line. Control values were taken from plants in rows near to the putative transgenic plants.

After 6 months, putative transgenic lines showed YLS between 4.5 and 2.5. In the control plants, the YLS of 'Navolean' was around 3.9. The evidence at four and five months evaluation revealed a decreasing YLS with the plant age. It was noted at six months, too. No differences between transgenic lines and the control were observed.

Table 2. Youngest leaf spotted (YLS) in 25 putative transgenic lines of 'Navolean' (Nv)/pHGA-91 at 4, 5 and 6 months after planting (First cycle, rainy season).

Lines	4 months	5 months	6 months
Nv 91-7	7.0 ± 0.0	6.0 ± 0.0	-
Nv 91-10	8.5 ± 0.71	6.0 ± 1.41	2.5 ± 0.70
Nv 91-11	7.4 ± 0.79	7.4 ± 1.27	4.0 ± 1.55
Nv 91-12	9.0 ± 1.87	7.5 ± 0.93	3.6 ± 0.52
Nv 91-14	8.2 ± 1.09	7.6 ± 1.59	3.8 ± 1.3
Nv 91-18	8.6 ± 0.73	8.1 ± 0.33	3.4 ± 0.73
Nv 91-19	5.5 ± 0.71	4.5 ± 0.71	-
Nv 91-22	9.0 ± 1.69	6.9 ± 1.05	3.6 ± 0.88
Nv 91-23	9.3 ± 1.12	8.4 ± 0.73	3.7 ± 1.11
Nv 91-26	7.3 ± 0.96	7.0 ± 1.00	-
Nv 91-28	9.2 ± 1.48	6.9 ± 1.21	3.3 ± 0.76
Nv 91-29	7.7 ± 1.32	7.4 ± 0.88	2.8 ± 0.41
Nv 91-31	8.3 ± 0.5	7.2 ± 0.84	3.0 ± 0.00
Nv 91-33	8.0 ± 1.10	7.3 ± 1.37	3.3 ± 0.81
Nv 91-40	9.0 ± 0.58	8.4 ± 1.19	3.9 ± 0.78
Nv 91-42	8.9 ± 0.33	8.6 ± 1.13	3.8 ± 0.83
Nv 91-43	8.4 ± 1.13	7.6 ± 0.73	3.2 ± 0.75
Nv 91-44	8.4 ± 0.98	8.1 ± 0.69	3.0 ± 1.40
Nv 91-52	6.5 ± 0.71	6.0 ± 0.0	-
Nv 91-53	8.0 ± 0.63	8.6 ± 0.55	4.5 ± 0.71
Nv 91-54	8.6 ± 0.88	7.8 ± 0.30	3.8 ± 0.75
Nv 91-56	8.9 ± 1.36	9.0 ± 0.76	4.0 ± 1.26
Nv 91-57	8.3 ± 0.71	8.2 ± 0.67	3.4 ± 0.74
Nv 91-58	8.1 ± 0.93	7.1 ± 0.93	3.7 ± 0.81
Nv 91-59	8.6 ± 1.24	7.3 ± 0.50	3.2 ± 0.98
Average	8.4 ± 1.18	7.7 ± 1.18	3.4 ± 0.94
Nv-Control	8.0 ± 1.77 ns	7.4 ± 1.46 ns	3.9 ± 1.46 ns
GN-Control	6.9 ± 0.78	6.3 ± 0.50	3.8 ± 0.46

Mean ± SD (Standard Deviation) of at least three replicates

Second evaluation in the field

The bunch emergence started in May. All plants of the same line had not flowered in June. The results of field evaluation in many putative transgenic plants at bunch emergence are showed in table 3.

Up to this point in the evaluation, one putative transgenic line with differences to the control was selected according to the infection index at bunch emergence (Table 4).

According to the results in the greenhouse as well as in field, a group of transgenic lines that present stages of maximum infection between 2 and 3 can be outlined (scale proposed by Alvarado-Capó *et al*., 2003) with artificial infection in greenhouse (data not shown). These manifested differences with the non-transgenic control in the greenhouse and field. However, taking into account the data of the variables evaluated up to the bunch emergence, much of these lines do not show these differences with the control. For this reason, Dr. Guzman of CORBANA (2004 personal communication), suggested that the variables that allow statistical differences are the infection index, and symptom evolution time, which should be included as the most important variable for the evaluations of the lines in field for next year (second cycle), so much for transgenic lines in second as well as the first evaluation cycle. Also the type of action of the quimeric genes in the cv. 'Navolean' have importance. These genes codified for protein accumulation in vacuole cell and they need the pathogen penetrates through the stomata of the leaf and begins the process of infection activating these genes. This explains much of the results obtained. Therefore, they will always appear in the supposedly resistant transgenic lines of first grades of infection of the disease.

Table 3. Reaction to black leaf streak disease in field at bunch emergence (shooting) of 15 putative transgenic lines of 'Navolean'/ pHGA-91.

Lines	No. pl.	NTL	YLS	NFL	II
Nv 91-1	3	11.0 ± 1.00	11.0 ± 1.00	11.00 ± 1.00	1.5 ± 0.14
Nv 91-14	3	11.0 ± 1.73	11.0 ± 1.73	11.0 ± 1.73	1.5 ± 0.27
Nv 91-18	8	11.6 ± 1.51	11.5 ± 1.51	11.5 ± 1.51	1.8 ± 0.97
Nv 91-22	6	10.5 ± 1.05	10.3 ± 1.03	10.5 ± 1.05	2.6 ± 1.82
Nv 91-23	5	12.6 ± 2.30	12.6 ± 2.30	12.6 ± 2.30	2.0 ± 1.36
Nv 91-29	2	9.50 ± 0.71	9.50 ± 0.71	9.50 ± 0.71	1.8 ± 0.13
Nv 91-40	4	11.2 ± 0.50	11.2 ± 0.50	11.2 ± 0.50	1.5 ± 0.06
Nv 91-42	7	10.4 ± 1.62	10.4 ± 1.62	10.4 ± 1.62	2.3 ± 1.14
Nv 91-43	2	12.5 ± 2.12	12.5 ± 2.12	12.5 ± 2.12	1.3 ± 0.22
Nv 91-44	2	11.0 ± 0.00	11.0 ± 0.00	11.0 ± 0.00	2.3 ± 1.05
Nv 91-53	2	12.5 ± 0.71	12.5 ± 0.71	12.5 ± 0.71	1.3 ± 0.07
Nv 91-54	7	11.0 ± 2.00	11.0 ± 2.00	11.0 ± 2.00	1.5 ± 0.26
Nv 91-56	4	13.0 ± 1.83	13.0 ± 1.83	13.0 ± 1.83	1.3 ± 0.18
Nv 91-57	2	13.0 ± 1.41	13.0 ± 1.41	13.0 ± 1.41	1.3 ±0.13
Nv 91-58	3	14.7 ± 3.06	14.7 ± 3.06	14.7 ± 3.06	1.5 ± 0.33
Average	4	11.6 ± 1.84	11.5 ± 1.84	11.6 ± 1.84	1.8 ± 0.92
Nv-Control	8	11.3 ± 2.14	11.3 ± 2.14	11.3 ± 2.14	2.3 ± 1.36
GN control	9	9.6 ± 1.73	9.0 ± 1.65	9.6 ± 1.73	3.6 ± 2.50

Mean ± SD of at least three replicates

NTL: number of total leaves youngest leaf spohed, NFL: number of functional leaves, II : Infection index.

Table 4. Putative transgenic lines selected of 'Navolean'/ pHGA-91 (1st Cycle, rainy season).

Lines	No. pl.	NTL	YLS	NFL	II
Nv 91-22	6	10.5 ± 1.05	10.3 ± 1.03	10.5 ± 1.05	2.6 ± 1.82
Nv 91-56*	4	13.0 ± 1.83	13.0 ± 1.83	13.0 ± 1.83	1.3 ± 0.18
Nv 91-57*	2	13.0 ± 1.41	13.0 ± 1.41	13.0 ± 1.41	1.3 ±0.13
Nv 91-58*	3	14.7 ± 3.06	14.7 ± 3.06	14.7 ± 3.06	1.5 ± 0.33
Nv- Control	8	11.3 ± 2.14	11.3 ± 2.14	11.3 ± 2.14	2.3 ± 1.36
GN control	9	9.6 ± 1.73	9.0 ± 1.65	9.6 ± 1.73	3.6 ± 2.50

NTL: number of total leaves youngest leaf spohed, NFL: number of functional leaves, II : Infection index.
**These lines have differences with the control according to agronomical characteristics.*

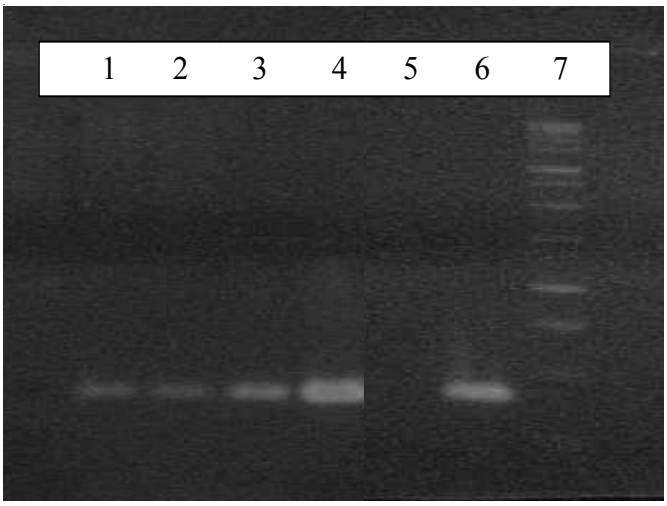

Figure 3. PCR analysis of putative transgenic lines of 'Navolean' using *bar* gene specific primers. Lane 1-3 Nv91-23, Nv91-40, Nv91-42; Lane 4: Plasmid pHCA58, positive control; Lane 5: Negative control; Lane 6: Positive control (Tobacco); Lane 7: Molecular weight marker 1 Kb.

From the total number of line (15), one was selected (6.6%) because it presented a superior response to *Mycosphaerella fijiensis* under field conditions when compared with the control of 'Navolean'. Osmotin (AP24) antifungal activity is synergistic with chitinase and glucanase *in vitro* and *in vivo* (Veronese *et al.,* 2003), which supports the results in this research. The Osmotin protein from tobacco causes perturbation in the regulation of fungal cell wall assembly (Yun *et al.,* 1998). These are the first results reported in plantain transgenic plants evaluated in field with antifungal proteins.

Molecular analysis

DNA isolation and PCR analysis

The extracted DNA was checked by an electrophoresis on agarose gel, confirming its purity and integrity.

The *bar* gene could be amplified in several transformed lines (Figure 3). The bands of the transformed lines were the same to those of the plasmid (pHCA58), which was used as positive control. In the negative control (non-transformed plants) no band appeared. Those indicated that the bands of the transformed lines corresponded to the *bar* gene, which was introduced into the genome of the plants by the transformation events that took place. Out or the five putative transgenic lines selected for molecular analysis, 4 (80.0 %) were positive in the PCR (Table 5).

Works carried out in this same crop by Remy *et al.* (2002) that regenerated a total of 157 *in vitro* differentiated plants of *Musa* spp., revealed the expression in eight individuals. Similar result was obtained by Daniels (2003), who achieved the expression in five of a total of seventy putative transgenic plants.

Table 5. PCR analysis for the presence of the *bar* transgene in putative transformed lines of 'Navolean' (pHGA-91) cultivar.

Cultivar	Construct	Line No.	*bar* PCR
Navolean	pHGA91	23	+
		40	+
		42	+
		53	-
		56	+
Total (+)		4	80 %

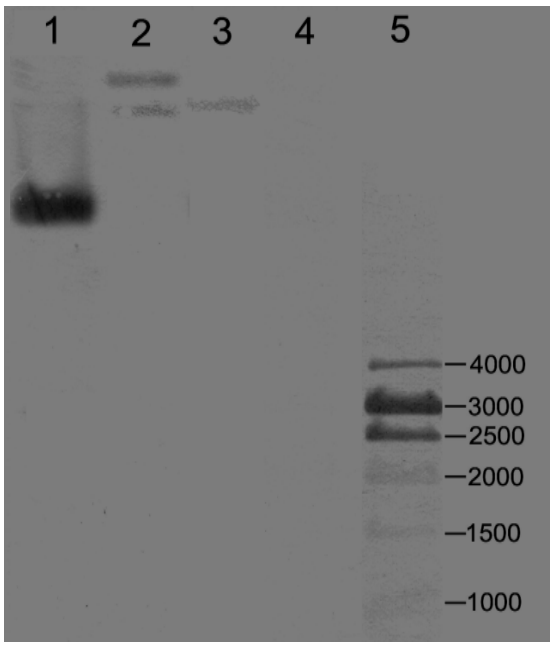

Figure 4. Southern blot to detect *bar* gene copy number, total DNA isolated from independent putative transgenic plants with a. Lane 1: Positive control Plasmid pHCA58; Lanes 2-3: Transformed lines; Lane 4: PCR product (402 bp); Lane 5: Molecular weight Marker (Mirus).

Southern blot analysis

All amplified DNA fragments reacted strongly with the *bar* specific probe when they were analyzed by Southern blot. Integration of the *bar* gene was further analyzed by genomic Southern blot DNA extracted from three putative transgenic lines that were PCR positive. These analysis in the three lines revealed that each one had a single-copy T-DNA (Figure 4). No hybridization was noted with the DNA extracted from the control. Similar results had been obtained when different crops were transformed by *Agrobacterium tumefaciens* with respect to the number of T-DNA insertion (Li *et al*., 2002; Tsukazaki *et al*., 2002; Lombari *et al*., 2003; Khanna and Daggard, 2003). The Southern blot analysis (Figure 4) confirmed the integration of the *bar* gene in two independent lines and shows one and two copies of the gene into the genome of these lines Nv 91-42 and Nv 91-56.

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