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Research article

Simplified methodology for large scale isolation of homozygous transgenic lines of lettuce

Flavia S. Darqui [a,b], Laura M. Radonic [a], Nilda López [a], H. Esteban Hopp [a,c], Marisa López Bilbao [a,*]

[a] Instituto de Biotecnología, CICVyA, Instituto Nacional de Tecnología Agropecuaria (INTA), Nicolás Repetto y De Los Reseros S/N, Hurlingham, Provincia de Buenos Aires, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas, Av. Rivadavia 1917, Ciudad Autónoma de Buenos Aires, Argentina
[c] Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Intendente Güiraldes 2160, Ciudad Universitaria, Ciudad Autónoma de Buenos Aires, Argentina

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ABSTRACT

Background: Lettuce is a globally important leafy vegetable and a model plant for biotechnology due to its adaptability to tissue culture and stable genetic transformation. Lettuce is also crucial for functional genomics research in the *Asteraceae* which includes species of great agronomical importance. The development of transgenic events implies the production of a large number of shoots that must be differentiated between transgenic and non-transgenic through the activity of the selective agent, being kanamycin the most popular.
Results: In this work we adjusted the selection conditions of transgenic seedlings to avoid any escapes, finding that threshold concentration of kanamycin was 75 mg/L. To monitor the selection system, we studied the morphological response of transgenic and non-transgenic seedlings in presence of kanamycin to look for a visual morphological marker. Several traits like shoot length, primary root length, number of leaves, fresh weight, and appearance of the aerial part and development of lateral roots were affected in non-transgenic seedlings after 30 d of culture in selective media. However, only lateral root development showed an early, qualitative and reliable association with *nptII* presence, as corroborated by PCR detection. Applied in successive transgenic progenies, this method of selection combined with morphological follow-up allowed selecting the homozygous presence of *nptII* gene in 100% of the analyzed plants from T2 to T5.
Conclusions: This protocol allows a simplified scaling-up of the production of multiple homozygous transgenic progeny lines in the early generations avoiding expensive and time-consuming molecular assays.

1. Introduction

Lettuce (*Lactuca sativa* L.) is a leafy vegetable that is globally grown and widely consumed [1]. It is also a model plant for biotechnology research since lettuce explants are highly responsive to a wide range of culture media in tissue culture, and regenerated shoots have been recorded for many genotypes [2]. The development of a stable transformation system in lettuce has enabled the introduction of many potentially useful genes in this crop, oriented to the molecular breeding of lettuce itself as well as to the production of molecules of economic interest. For example, lettuce has been transformed with *AtHSP17.8*, an *Arabidopsis thaliana* gene coding for a heat shock protein, to confer resistance to abiotic stresses [3]. This model plant has also been selected as a platform for recombinant production of miraculin, a taste-modifying glycoprotein extracted from the red berries of the West African native shrub *Richadella dulcifica* [4]. Lettuce has advantages for biotechnology applications, for instance, it can be eaten fresh allowing the preservation of labile functions susceptible to storage denaturation. Its adaptability to greenhouse conditions and hydroponic culture allows cultivation in controlled environments that can be easily scaled up or down to grow almost anywhere. As a plant bioreactor, its life cycle is shorter than other plant alternatives.

Lettuce is also crucial for the progress of functional genomics research in *Asteraceae*, which is the largest plant family on earth, with over 24,000 species described, representing almost 10% of all flowering plant species [5]. It includes economically important crops (over 40 species have been domesticated for a wide variety of uses), nice wildflowers, weeds and several species containing molecules of medical interest [6]. Due to its rather large genetic distance to *Arabidopsis*, lettuce became a much reliable model system for understanding the functionality of the emerging genomic knowledge of this family. For example, for a typical experiment aimed to characterize a sunflower gene function, it is not only possible to overexpress a given gene sequence, but also to knock out or down a homologous gene sequence, which is sometimes impossible in *Arabidopsis* due to divergent evolution.

* Corresponding author.
E-mail address: lopezbilbao.marisa@inta.gob.ar (M. López Bilbao).
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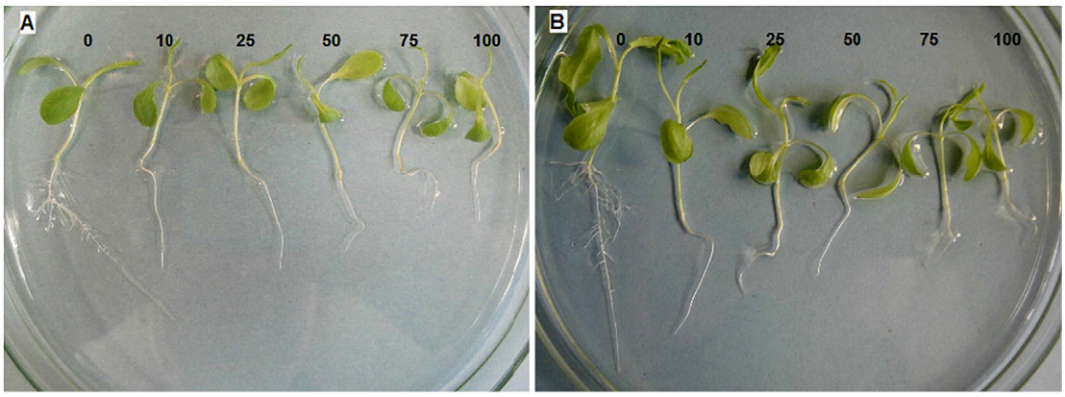

Fig. 1. Non-transgenic seedlings germinated at different concentrations of km. Seedlings cultured in 1/2 MS supplemented with 0, 10, 25, 50, 75 and 100 mg/L Km during 3 (A) and 7 d (B).

Functional genomics of many unknown gene functions requires a scaled up method for the analysis of multiple transformation events derived from multiple gene transgenesis assays. To this aim, it is necessary to simplify the steps towards the obtaining of stabilized homozygous transgenic lines through selfing for several generations. Molecular analysis by Southern blot, PCR or nucleotide sequencing in early generations like T0 or T1 is not only costly and time-consuming but also may not be efficient for distinguishing heterozygous from homozygous plants, especially when multiple transgene copies are incorporated in the genome [7]. Standard molecular methods may not be sufficient to predict and/or detect transcriptional silencing of the transgenes after the first or second generation. Transient somatic variation due to epigenetic modifications during tissue culture conditions that may affect the early transformed generations are not detected either.

The *neomycin phosphotransferase II* gene (*nptII*) is the most widely used selectable marker for plant transformation [8]. Plants such as maize, cotton, tobacco, Arabidopsis, flax, soybean and many others have been successfully transformed with *nptII*. Lettuce has also been transformed with this gene by several groups [9,10,11,12]. This gene codes for aminoglycoside 3′-phosphotransferase, which inactivates aminoglycoside antibiotics such as kanamycin, neomycin and gentamycin by phosphorylation [13], being kanamycin (Km) the most used one. Kanamycin is known to interact with the 30S subunit of chloroplast and mitochondrial ribosomes, disrupting protein synthesis and photosynthesis. Sensitivity of plants to a selective agent depends on many factors including: species, explant type, developmental stage, and tissue culture conditions [14]. The effectiveness of an antibiotic resistance system also depends on defining the lowest concentration of antibiotic that suppresses growth of non-transgenic plants but does not cause detrimental effects to transgenic ones. However, sometimes it is not possible to ascertain a concentration of the selective agent that completely kills non-transgenic plants without significantly affecting the viability of the transgenic plants. Besides, the development of a transgenic event with an agronomical advantage implies that a large number of transgenic shoots must be produced and advanced in several generations to obtain homozygous lines for testing in field trials. So, it is very important to design a direct *in planta* assay focused on the activity of the selective gene which enables a rapid, easy and cost-effective characterization of a large number of plants during the evaluation of the segregating progeny. In this context, it is necessary to find a diagnostic morphological marker that helps to adjust a selection pressure strong enough to avoid escapes but without affecting transgenic plants viability. There are reports of such phenotypic markers. In the case of *Arabidopsis thaliana* transformed by floral dip with *nptII* gene, selection is performed in plantlets germinated in Km, where transformed seedlings have green expanded cotyledons whereas non-resistant ones have pale unexpanded cotyledons [15]. Another simple scheme for evaluation of transgenic plants was developed for testing sorghum plants containing the *bar* gene [16] in which T1 plants were characterized by the chlorosis of leaves painted with a solution of glufosinate ammonium.

In the case of lettuce, the effect of kanamycin on explant performance for regeneration of viable transgenic T0 shoots is very well documented [2]. However, to our knowledge, there are no thorough reports on the morphological effects of kanamycin on transgenic germinating seedlings.

In the present work, we identify a morphological feature displayed only by transgenic plants germinated in selective medium, which can enable accurate selection of Km-resistance seedlings. We define the optimal concentration of Km to select transgenic lettuce plants *in vitro,* with no escapes and no need of any further molecular test in the early generations.

2. Materials and methods

2.1. Plant material

Lettuce (*Lactuca sativa* var. 'Grand Rapids') seeds were originally provided by the seed bank of the Estación Experimental Agropecuaria "La Consulta", INTA, Mendoza, Argentina.

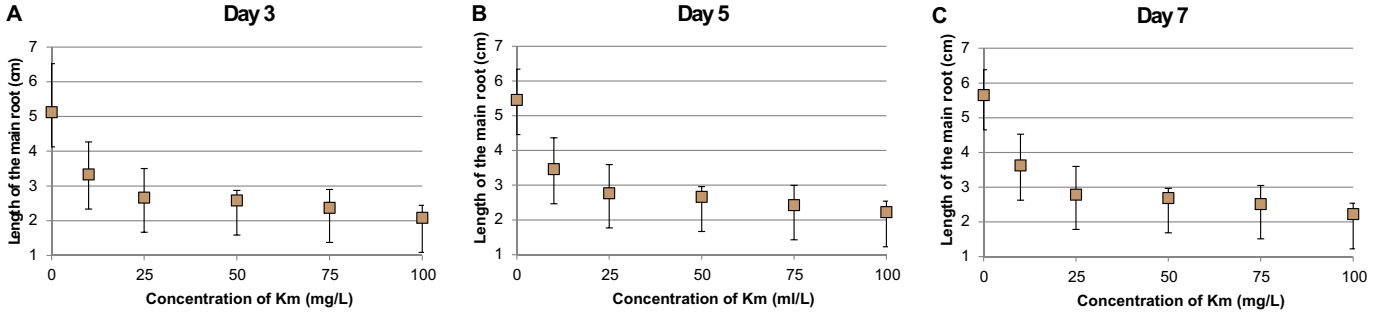

Fig. 2. Length of the main root of non-transgenic seedlings germinated at different concentrations of Km. Average length of the main root of non-transgenic seedlings germinated in 1/2 MS supplemented with 0, 10, 25, 50, 75 and 100 mg/L of Km at 3(A), 5(B) and 7 d(C). ANOVA p value <0.0001. Bars indicate standard deviations.

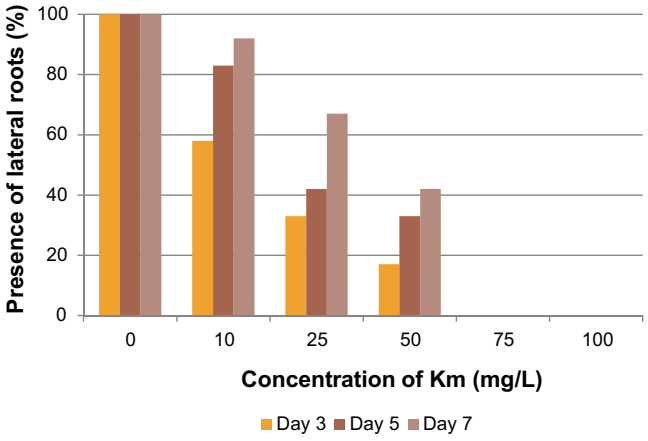

Fig. 3. Development of lateral roots in non-transgenic seedlings germinated at different concentrations of Km. Percentage of non-transgenic seedlings with development of lateral roots, germinated in 1/2 MS containing 0, 10, 25, 50, 75 and 100 mg/L of Km, at 3, 5 and 7 d.

Transgenic seeds used in these experiments came from four different T0 events (1.7, 2.2, 4.1, 4.2.2) carrying the complete sequence of *snakin-1* gene [17] and *nptII* gene. Genetic transformation of lettuce was performed following the protocol established by Curtis et al. [18] and modified by our group. Young leaves were transformed by co-cultivation with the LBA4404 *Agrobacterium tumefaciens* strain harboring the pK7WG-*rbcS1* vector. This vector was developed from the vector pK7WG2 [19], with the addition of *rbcS1* promoter instead of *CaMV35S* promoter. Transgenic shoots were selected in Petri dishes containing half strength MS [20] basal medium (1/2 MS) with 10 g/L sucrose and 0.8% agar, supplemented with 50 mg/L kanamycin. Afterwards, selected shoots were taken to the greenhouse. T0 plants were analyzed through PCR and selfed to obtain T1 progeny. Four T1 plants from each T0 event were PCR-confirmed and named with the number assigned to its T0 plus a letter to differentiate the lines emerged from the same event. T1 plants were self-pollinated to obtained T2 lines. Although lettuce is autogamous, a possible cross-pollination was prevented by covering flowers with a fabric bag.

Non-transgenic control seeds were obtained from non-transformed plants. These plants were cultured *in vitro* exactly as the transformed ones, except that in the co-culture step they were cultured in absence of *Agrobacterium*. Greenhouse conditions were the same for both transgenic and non-transgenic plants.

Thirty day-old *in vitro* seedlings (both transgenic and non-transgenic) were taken to the greenhouse and acclimatized during 48 h. Next, they were transplanted to 350 mL pots and covered with a transparent plastic bag during 72 h to be adapted to humidity conditions. Soil composition was 2 parts of peat, 2 parts of pine bark compost, 1 part of perlite and 1/4 part of organic amendment. Plants were fertilized with 1.5 g/L of Hakaphos NPK 18-18-18 (Compo Expert, Germany) three times per week. Plants were maintained under these conditions (18–22°C, 8/16 h dark/light cycle) until seed production.

In all tests described herein, the transgenic and non-transgenic seeds came from plants of the same age and physiological stage, harvested at the same time and kept under the same storage conditions.

2.2. Molecular analyses

For PCR assays, genomic DNA was extracted from leaf tissue by CTAB method [21]. PCR analysis was done to confirm the integration of both the *snakin-1* gene and the selection cassette. Primers were designed according to the *snakin-1* gene and the *nopaline synthase* promoter (Pnos) sequence (which ruled the expression of *nptII* gene and was downstream of the *snakin-1* cassette in the DNA sequence): forward primer (5′ TTCAGCTCGAGAAAAAATGAAGTTATTTCTATTAACT 3′); reverse primer (5′ GCCTCGATCGAGTTGAGAGTGA 3′).

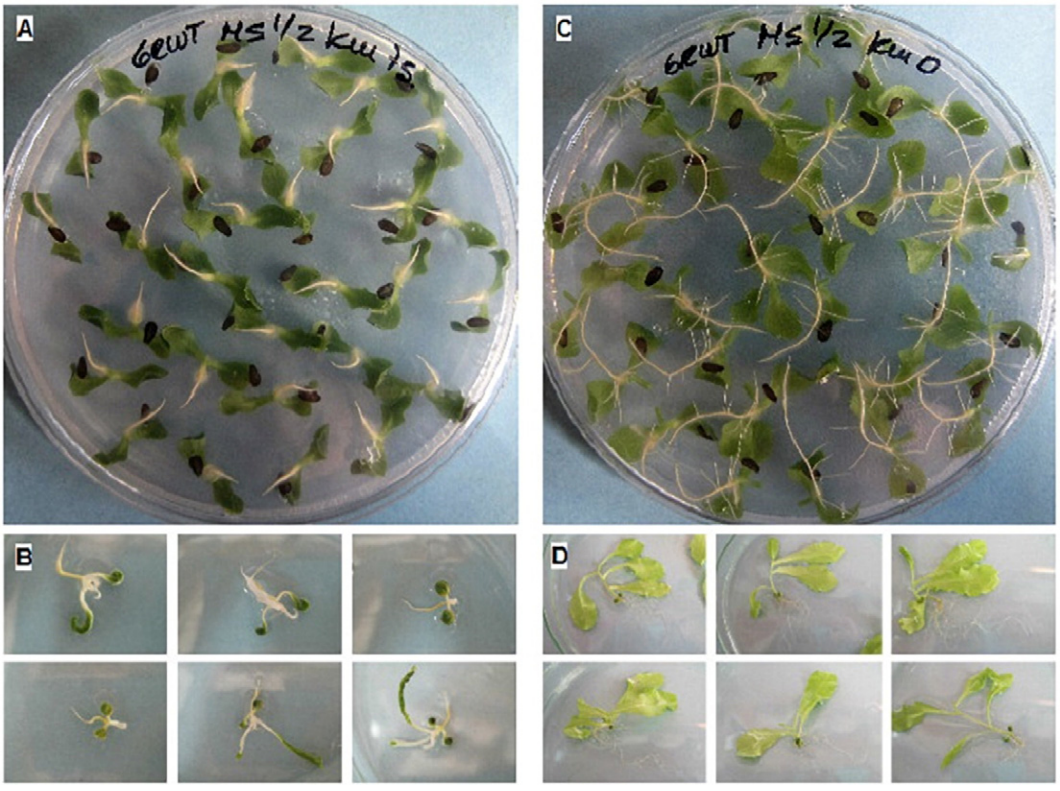

Fig. 4. Non-transgenic seedlings germinated in presence or absence of km. 10 d non-transgenic seedlings cultured in 1/2 MS + 75 mg/L Km (A) or in 1/2 MS without Km (C). 30 d non-transgenic seedlings cultured in 1/2 MS + 75 mg/L Km (B) or in 1/2 MS without Km (D).

A 50 μL reaction mixture was prepared, containing 60 ng of template DNA, 1× buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 μM of each primer and 2.5 units of Taq DNA polymerase. Amplification was carried out using Programmable Thermal Controller-100 Thermal Cycler (M. J. Research Inc., USA). After one cycle at 94°C for 2 min, samples were subjected to 5 cycles in a touch down program (94°C for 30 s, 60°C for 60 s and 72°C for 90 s, annealing temperature decreasing 1°C in each cycle), then performed 30 cycles (94°C for 30 s, 55°C for 60 s and 72°C for 90 s) ending with a final extension at 72°C for 10 min. Amplified products corresponded to 1401 bp fragments and were visualized by agarose (0.8%) gel electrophoresis.

For Southern blotting assay, genomic DNA was extracted from T3 plants, following the protocol for high latex content plants described by Michiels et al. [22]. A DNA extraction from a non-transgenic plant was used as a negative control. Twenty micrograms of DNA were digested with BamHI and subjected to agarose (0.8%) gel electrophoresis. DNA was transferred by capillarity to an Amersham Hybond XL Nylon Membrane (GE Healthcare Life Sciences, UK), using SSC 10× buffer, pH 7. Later DNA was fixed to the membrane by exposure to a UV light source with an energy of 0.012 J/cm^2.

The radioactive probe was prepared using Prime-a-Gene Labeling Kit System (Promega, USA) according to the manufacturer employing α-[^{32}P]-dCTP, random primers and *nptII* sequence as template. Hybridization procedures were carried out as described by Green and Sambrook [23]. Membrane was washed twice in 2× SSC-0.1% SDS for 10 min at room temperature. Washed membrane was exposed overnight on an Imaging Plate (Fujifilm, Japan). The plate was then scanned using Amersham Typhoon Trio Variable equipment Mode Imager (GE Healthcare Life Sciences, UK).

2.3. Evaluation of seedlings germinated in selective media

To assess morphologic characteristics featured by non-transgenic seedlings growing in presence of Km, sterilized non-transgenic seeds were sown in 1/2 MS supplemented with increasing concentrations of Km and taken to a growing chamber (23°C, 16 h light/8 h dark). Sample size was 12 seeds per treatment. Each treatment consisted in the supplementation with increasing concentrations of Km (0, 10, 25, 50, 75 or 100 mg/L) for different culture times (3, 5 or 7 d). After treatments, aspect of the aerial part, number of leaves, length of the main root, and presence/absence of lateral roots were observed in all non-transgenic seedlings. Percentage of plants with presence of lateral roots was calculated for each group. Regarding length of the main root, data was analyzed using Infostat Software [24] and average and standard error values were calculated for each treatment and compared in a scatterplot.

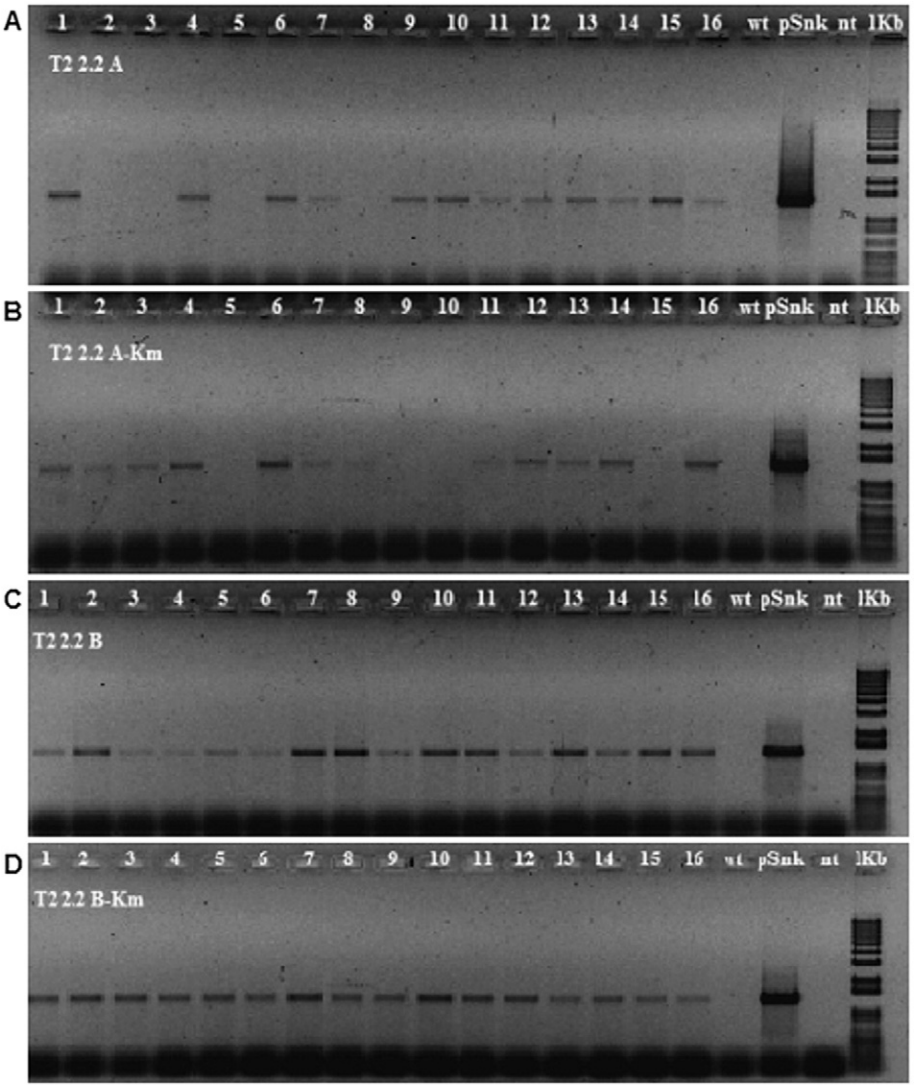

Fig. 5. PCR results of lines 2.2A and 2.2B. Amplification products of PCR assays performed in seedlings from line 2.2A germinated in absence (A) or presence (75 mg/L) of Km (B) and from line 2.2B germinated in absence (C) or presence (75 mg/L) of Km (D).

Later, development of lateral roots was evaluated in a much larger sample of non-transgenic seedlings and for a longer period of time. Sixty non-transgenic seedlings were germinated in 1/2 MS or in 1/2 MS + 75 mg/L Km. Once cotyledons arose, seedlings were transferred to 200 mL glass jars containing 20 mL of medium. Each jar contained 4 plantlets. Shoots were maintained in a growing chamber during 30 d with medium renewal every 10–14 d. After treatments, the presence/absence of lateral roots was visually screened in all seedlings.

Next, T2 seedlings from different T1 lines (considering each T1 plant, derived from a particular T0 event, as a separated line itself, since it could be homozygous or hemizygous for the transgene) were germinated in presence/absence of Km, after being sterilized as previously indicated. A total of 128 transgenic seeds belonging to 4 different T2 independent lines (1.7D, 2.2A, 2.2B and 4.1B) and 32 non-transgenic seeds were germinated in absence of Km or in presence of 75 mg/L Km in the growing media (16 seeds/line/treatment). When cotyledons arose, seedlings were transferred from Petri dishes to glass jars and maintained in a growing chamber during 30 d, as previously described.

Different parameters were observed to find out if there were morphological responses due to the presence of *nptII* transgene and to differentiate transgenic seedlings from non-transgenic ones. Some of these parameters were quantitative (fresh weight, length of the stem, number of leaves and length of the main root) and others were qualitative (seedling appearance and presence of lateral roots). All seedlings were processed for DNA extraction and PCR assays. Each transgenic line was analyzed separately, considering that the response of plants to Km may differ between lines due to gene expression level, transgene site of insertion, number of integrated copies, transgene homozygosis or heterozygosis, *etc.*

All seedlings were also evaluated through PCR to confirm presence of *nptII* transgene. Statistical analyses were performed using InfoStat Software. Quantitative variables were studied through ANOVA and Tukey tests. Qualitative variables were analyzed through a Pearson's chi-square test in contingency tables. Concerning to the dependent variable "appearance", plantlets were ranked in three categories: "good" (plants look healthy and bright green), "affected" (green color not as intense or abnormal shape or texture of leaves) or "very affected" (very pale or completely white). Regarding to the dependent variable "presence of lateral roots", plantlets were ranked in two categories: "yes" (the plant shows presence of lateral roots), or "no" (the plant does not show any lateral root). The independent variable was the presence (75 mg/L) or absence (0 mg/L) of Km in germination medium.

3. Results

3.1. Major morphological effects of kanamycin in the development of non-transgenic lettuce seedlings

Non-transgenic seedlings germinated at different concentrations of Km containing medium (0, 10, 25, 50, 75 or 100 mg/L) were observed at 3, 5 and 7 d. Until the seventh day of culture, no differences were noticed in seedlings cultured at different concentrations of Km in terms of color, size or number of leaves (Fig. 1). However the increasing concentrations of Km affected directly the length of the primary root (Fig. 2).

Although Km affected drastically the length of the primary root even at low concentrations of Km (25 mg/L), no significant differences were observed between seedlings grown in the range of 25 to 100 mg/L of Km (Fig. 1 and Fig. 2). In addition, as it will be shown below, Km affects this trait not only in non-transgenic seedlings but also in transgenic seedlings. This morphological marker turned out to be unreliable to detect the cut off concentration of Km needed to avoid non-transgenic escapes to selection.

Besides, the increasing concentrations of Km influenced the development of lateral roots. All seedlings cultured in absence of Km developed lateral roots very soon (by the third day of culture). Conversely, all seedlings cultured at 75 mg/L Km showed total absence of lateral roots, even after 7 d of culture (Fig. 3).

Moreover, from 60 non-transgenic seeds germinated in 1/2 MS with 75 mg/L Km, none of the emerging seedlings showed any lateral roots after 30 d of culture (Fig. 4A). At the same time, from 60 control seedlings germinated in 1/2 MS without Km, all developed typical and normal lateral roots (Fig. 4C). These results confirmed that absence of lateral roots was an unequivocal morphological response featured by all non-transgenic shoots when they were germinated in presence of Km. Thus 75 mg/L was established as the threshold concentration of Km.

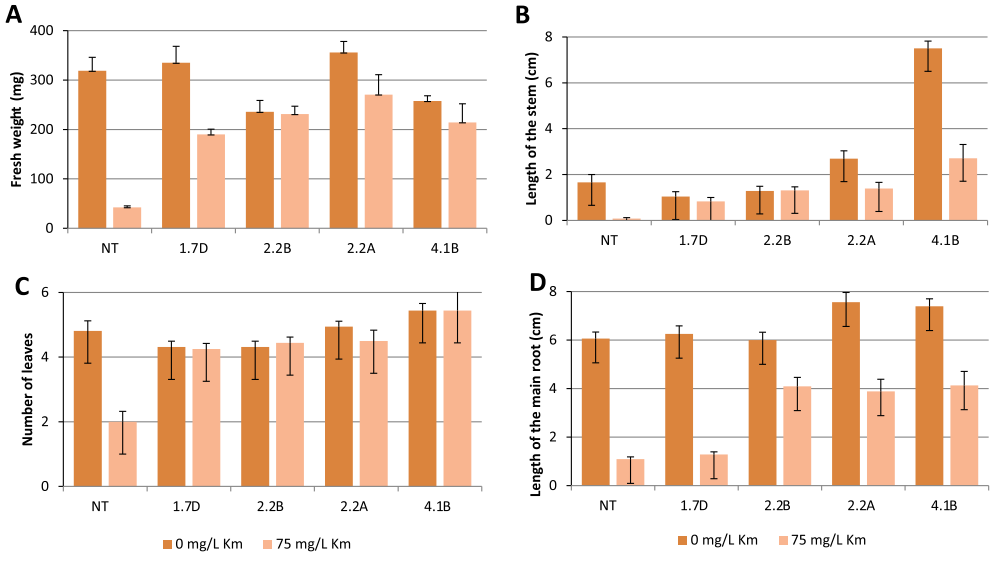

Fig. 6. Morphological variables analyzed in transgenic and non-transgenic seedlings cultured in medium with or without Km. Comparison of fresh weight (A), length of the stem (B), number of leaves (C) and length of the main root (D) in non-transgenic (NT) and transgenic PCR-positive T2 plants germinated in 1/2 MS (0 mg/L Km) or in selective medium (75 mg/L Km) during 30 d. ANOVA *P*-value <0.0001 (for each variable).

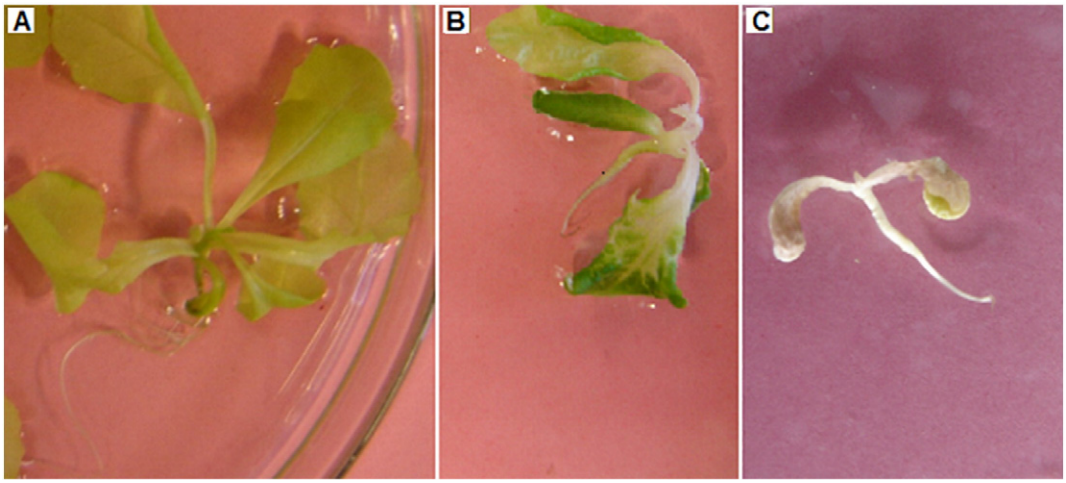

Fig. 7. Km effect in appearance of plantlets. Plantlets were categorized as good (A), affected (B) or very affected (C).

3.2. Presence of lateral roots as a reliable morphological marker for selecting transgenic seedlings with no escapes

Several differential morphological responses to Km between transgenic and non-transgenic seedlings were analyzed. All plantlets from the non-transgenic line were PCR-negative, as expected. All plantlets from line 1.7D and line 2.2B were PCR-positive while in lines 2.2A and 4.1B some seedlings were PCR-negative. As an example, PCR results of lines 2.2A and 2.2B are shown in Fig. 5.

After 30 d of culture, all morphological variables analyzed were influenced by the presence of Km in non-transgenic seedlings (ANOVA p-value <0.001 for each variable, Fig. 6).

In transgenic T2 plants confirmed by PCR, most lines showed no differences in fresh weight between treatments, except for line 1.7D, with lighter plants in presence of Km. Length of the stem in plants from lines 2.2A and 4.1B was significantly affected by the presence of Km, but in lines 1.7D and 2.2B no effect of the antibiotic was detected on this trait. In brief, regarding fresh weight and length of the stem, different transgenic lines showed dissimilar responses to Km. Concerning the length of the main root, transgenic and non-transgenic lines displayed the same response; plants had shorter main roots when they were germinated in selective medium. Thus, none of these parameters was adequate to differentiate transgenic from non-transgenic seedlings germinated in selective medium. Finally, the presence of Km did not affect the number of leaves in any transgenic line, while it did affected the number of leaves in non-transgenic plants (Fig. 6).

Regarding the appearance (Fig. 7), transgenic lines showed different responses to Km (Table 1). In Pearson's chi-square tests, plants from lines 1.7D ($p = 0.0028$) and 4.1B ($p = 0.0915$) were affected by the presence of Km in culture media, while plants from lines 2.2A ($p = 0.1249$) and 2.2B ($p > 0.99$) showed no significant differences between treatments. Consequently, "appearance" could not be considered the best parameter to select transgenic plants *in vitro*.

All PCR-positive seedlings developed lateral roots in selective media (Table 1). The presence of *nptII* always correlated with the presence of lateral roots (Table 2). No escapes were observed, *i.e.*, none of the PCR-negative plants developed lateral roots. We concluded that "presence of lateral roots" was the only reliable parameter to determine the presence of *nptII* and Km-resistance.

3.3. Analysis of segregation of nptII transgene

To evaluate if lateral root development in selective media was a useful criterion to analyze transgene segregation, we study transgenic progenies from T0 to T5.

T1 seeds obtained from different T0 events (1.7, 2.2, 4.1 and 4.2.2), were germinated in selective media (75 mg/L Km). T1 seedlings were classified as Km-resistant or Km-susceptible, according to the presence or absence of lateral roots. These seedlings showed a 3:1 phenotypic ratio of Km-resistance as confirmed through a chi-square test ($p < 0.01$; Table 3).

T1 seedlings, with lateral roots in Km and PCR-confirmed, were transferred to the greenhouse. They were named with the number assigned to its T0 plus a letter to differentiate the lines emerged from the same event. For example, four T1 seedlings coming from the T0 event 1.7, gave rise to T1 lines 1.7A, 1.7D, 1.7C and 1.7D. Since Km-resistant T1 seedlings could be in homozygosis or hemizygosis of the *nptII* gene, T2 seeds, from different self-pollinated T1 plants, were germinated and the Km-resistance phenotype was scored likewise. In this analysis, we included seeds from some of the T2 generations (T2 lines 1.7D and 2.2B) already used to determine the effect of Km in transgenic seedlings and other T2 generations. Table 4 indicates only

Table 1
Percentage of plantlets with good, affected or very affected appearance and percentage of plantlets with presence of lateral roots in medium with or without Km.

Treatment	Line[a]	Appearance			Presence of lateral roots	
		Good	Affected	V. affected	Yes	No
1/2 MS	1.7D	100%	–	–	100%	–
0 mg/L Km	2.2B	100%	–	–	100%	–
	2.2A	100%	–	–	100%	–
	4.1B	100%	–	–	100%	–
	non-transgenic	100%	–	–	100%	–
1/2 MS	1.7D	56%	44%	–	100%	–
75 mg/L Km	2.2B	100%	–	–	100%	–
	2.2A	100%	–	–	100%	–
	4.1B	80%	20%	–	100%	–
	non-transgenic	–	–	100%	–	100%

[a] Only T2 PCR-positive and non-transgenic plantlets were evaluated.

Table 2
Presence of lateral roots and PCR results in non-transgenic and transgenic T2 plantlets germinated in selective medium (75 mg/L Km).

Line[a]	Presence of lateral roots		PCR result	
	Yes	No	Positive	Negative
1.7 D	16	–	16	–
2.2 B	16	–	16	–
2.2 A	13	3	13	3
4.1 B	10	6	10	6
non-transgenic	–	16	–	16

[a] Both T2 PCR-positive, T2 PCR-negative and non-transgenic plantlets were evaluated.

Table 3
Segregation analysis of Km-resistance in T0 progeny.

T0 event	T0 progeny (T1 generation)[a]		$\chi^{2,b}$
	Km-resistant plants	Km-susceptible plants	
1.7	27	11	0.32
2.2	20	14	4.75
4.1	27	8	0.47
4.2.2	27	9	1.33

[a] Seeds obtained by selfing of the T0 transformants were germinated on selective medium (75 mg/L of Km).
[b] $\chi_{3:1} > 6.63$: significantly different from the hypothesis of a single insertion ($p < 0.01$).

Table 4
Segregation analysis of homozygous T2 Km-resistant generations.

T1 plant	T1 progeny (T2 generation)[a]	
	Km-resistant plants	Km-susceptible plants
1.7A	124 (98.4%)	2 (1.6%)
1.7D	120 (100%)	0 (0%)
2.2B	122 (98.4%)	2 (1.6%)
4.1A	50 (98%)	1 (2%)
4.2.2C	53 (98%)	1 (2%)

[a] Seeds obtained by selfing of T1 plants were germinated on selective medium (75 mg/L of Km).

T2 generations in which almost all seedlings showed Km-resistance as expected from the decrease of the proportion of segregating transgenic hemizygous plants after a second round of selection.

These Km resistant T2 generations were analyzed by PCR and all of them resulted positive for the *nptII* transgene presence. As an example, the PCR results of T2 1.7D seedlings can be seen in Fig. 8.

Homozygosis of selected T2 Km-resistant progenies was confirmed in the T3 generation following the same procedure. More than one-hundred seeds of each transgenic line were germinated in selective media. All of them developed lateral roots and presence of the *nptII* transgene was PCR-confirmed in these seedlings.

Characterization of the transgenic lines was done by Southern blot analysis (Fig. 9). It showed that lines 1.7A and 1.7D (which corresponded to the same transgenic event) and line 2.2B resulted to be multiple insertion events, with four and two copies respectively, while lines 4.1A and 4.2.2C were single insertion events. Despite the difference in the copy number of transgenes in each transgenic line, no differences were observed between them in response to Km, since seedlings from those lines with the highest number of insertions did not develop lateral roots earlier, or in larger numbers compared to seedlings from single copy lines.

The selection procedure described above was also done in T4 and T5 progenies to confirm presence and stability of *nptII* gene and homozygosis of lines. As an example, all T4 plants germinated in selective medium developed lateral roots (Fig. 10).

4. Discussion

Well known phenotypic consequences of kanamycin effect on plants are etiolation of aerial parts, arrest of growth and, at some point, the death of plants [25]. In other plants, the only reliable selection criterion is root formation, like in sunflower [26], a member of the Asteraceae family. In the case of lettuce, also an Asteraceae, there are scarce reports on seedling development parameters in presence of Km. Here we observed both, short and long term effects of Km on lettuce plants. In the short term (up to 7 d after germination) color, size and number of leaves did not show any major changes at concentrations of Km up to 100 mg/L (Fig. 1). In contrast, length of the root and emergence of lateral roots did certainly show significant differences, as early as 3 d after germination. Interestingly, the presence of Km in culture medium affected the development of roots before the presence of any symptoms in the aerial part of non-transgenic lettuce seedlings. These last results were not unexpected since there are reports showing similar effects in other systems like in the model plant *Arabidopsis thaliana* [27,28]. In these reports, the development of roots in *A. thaliana* seedlings was evidently affected by the concentration of Km in culture medium and lateral roots were completely absent in seedlings cultured at 50 mg/L Km.

In lettuce seedlings, several phenotypic effects became evident in their fresh weight, length of the stem and appearance after 30 d of cultivation in presence of Km (Fig. 6). However, these traits showed variation between different transgenic lines or were affected in both non-transgenic and transgenic seedlings. Instead, non-transgenic seedlings had fewer leaves in Km-selective medium than in antibiotic free-medium, while transgenic seedlings had no differences in the number of leaves between treatments. Nevertheless, it would not be the most suitable criterion for the selection of homozygous transgenic lines. To do so, it would be necessary to compare, on each transgenic line, two sets of seedlings (germinated in absence and presence of Km), and then counting the leaves to confirm if there are significant differences between treatments. This trait could not be individually evaluated on each seedling, as it should be analyzed in a group of seedlings for each treatment.

The emergence of lateral roots was still a reliable trait for selection after 30 d, when all transgenic seedlings were able to develop lateral roots in selective media. Besides, the presence of *nptII* in the genome always correlated with the presence of lateral roots and no escapes were observed. Thus, we concluded that development of lateral roots was the only morphological trait that confirmed the expression of *nptII,* determined as Km-resistance, in lettuce seedlings.

Our group has previously proved in sunflower, that the capability for rooting in Km-selective medium was the only useful criterion for

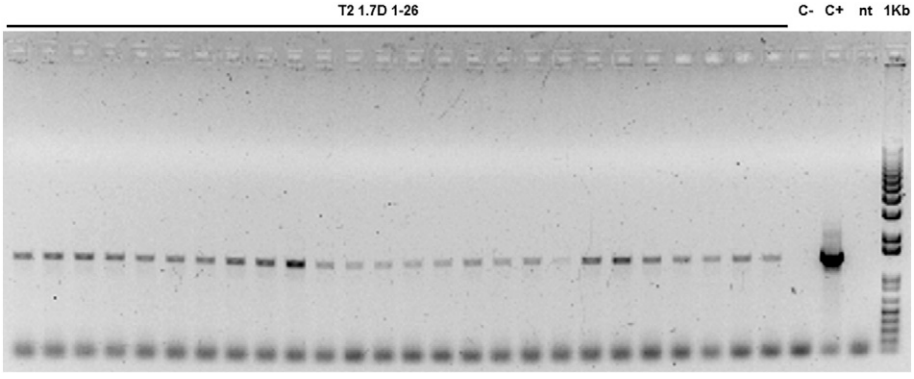

Fig. 8. PCR results of T2 1.7D seedlings. Amplified products from PCR assay performed in T2 1.7D seedlings (lane 1 to 26). C-: non-transgenic seedling. C+: PCR positive control (pK7WGR vector). nt: non-template control. 1Kb: 1 Kb Plus DNA Ladder (Invitrogen, USA).

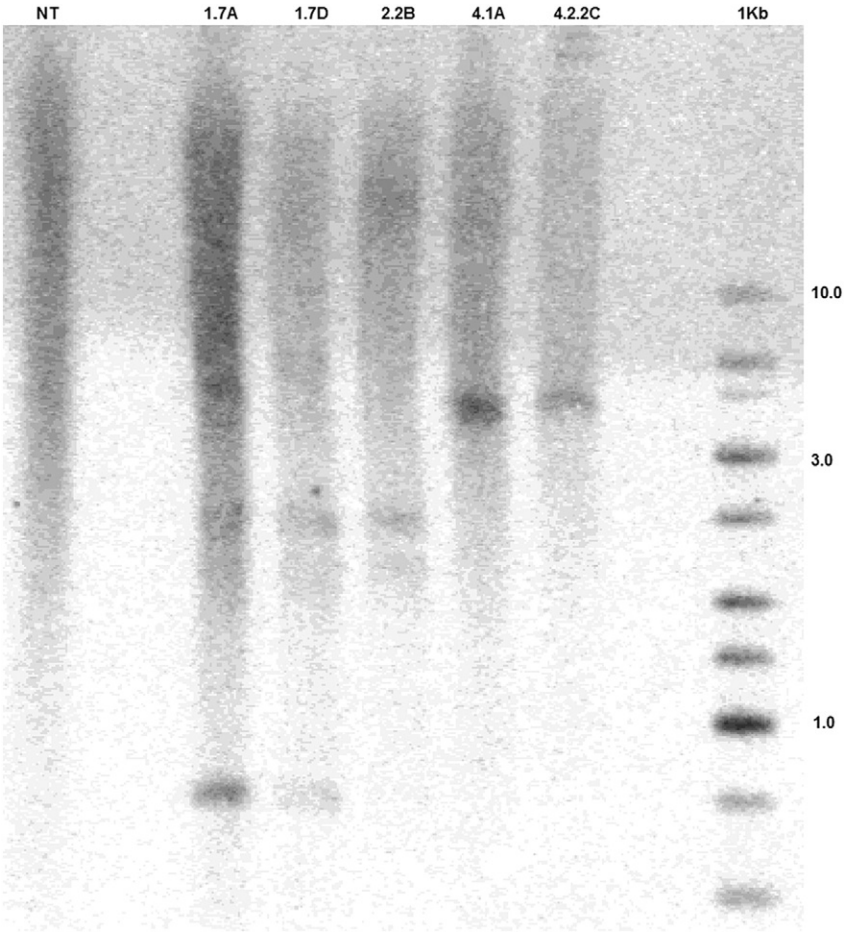

Fig. 9. Southern blot analysis performed in T3 lines. NT: non-transgenic line. 1Kb: 1Kb DNA Ladder (Fermentas). A probe corresponding to *nptII* sequence was used.

selecting transgenic shoots, since the color or aspect of shoots were not valid physiological parameters for identification of transgenic events [26].

To our knowledge, the present work is the first one to report the development of lateral roots as a reliable criterion for selection of transgenic lettuce plants *in vitro*. We developed a simple and accurate method, avoiding expensive and time-consuming PCR assays in every cultured plant. Thus, lateral root development can be a reliable tool to facilitate the phenotypic segregation of transgenic lettuce progeny for the obtainment of homozygous lines.

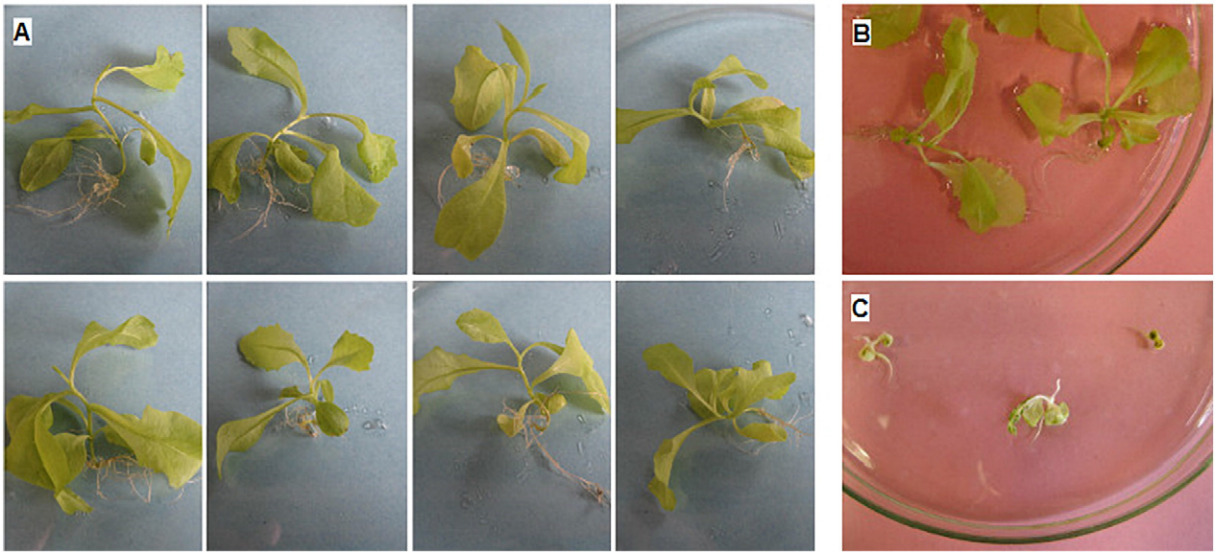

Fig. 10. Thirty day-old transgenic seedlings germinated in selective medium (75 mg/L Km) and 30 day-old non-transgenic control seedlings. Thirty day-old seedlings from line T4 2.2B germinated in selective medium (A). Thirty day-old non-transgenic seedlings germinated in non-selective medium (B). Thirty day-old non-transgenic seedlings germinated in selective medium (B).

Conflict of interest

Authors declare that there is no conflict of interest.

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