Electronic Journal of Biotechnology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

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

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ARTICLE INFO

Article history:
Received 8 June 2017
Accepted 29 September 2017
Available online xxxx

Keywords:
Homozygosis
Kanamycin
Lactuca sativa L.
Lines
nptii
Plants
Primers
Root
Seed
Seedling
Selection

ABSTRACT

Background: Lettuce is a worldwide cultivated and consumed leafy vegetable and a model plant for biotechnology, due to its adaptability to tissue culture and to stable genetic transformation. Lettuce is also crucial for functional genomics research in *Asteraceae*, the largest plant family that includes species of great agronomical importance. Kanamycin is the most popular selective agent for *in vitro* selection of transgenic shoots expressing *nptII* genetic marker.
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 the presence of kanamycin to look for a visual morphological marker. Several traits such as shoot length, primary root length, number of leaves, fresh weight, 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 the 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 plants analyzed 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], and 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 freshly 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

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2017.10.002

Please cite this article as: Darqui FS, et al, Simplified methodology for large scale isolation of homozygous transgenic lines of lettuce, (2017), https://doi.org/10.1016/j.ejbt.2017.10.002

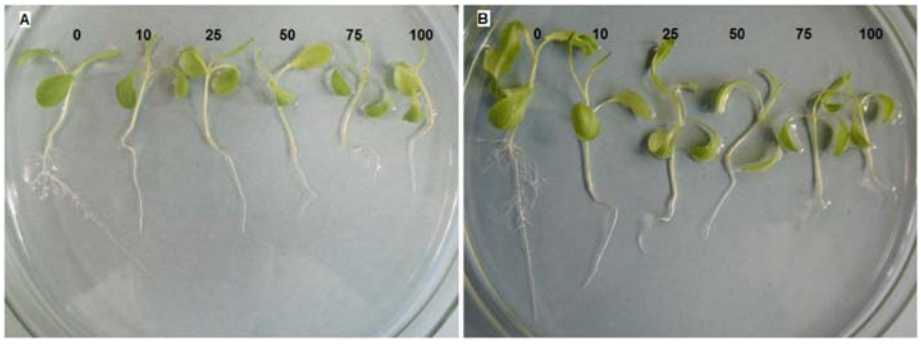

Fig. 1. Non-transgenic seedlings germinated at different concentrations of Km. Seedlings cultured in 1/2 MS supplemented with 0, 10, 25, 50, 75 and 100 mg/L Km during 3 (A) and 7 d (B).

overexpress a given gene sequence, but it also is to knock out or down a homologous gene sequence, which is sometimes impossible in *Arabidopsis* due to divergent evolution.

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. 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 [7]. Plants such as maize, cotton, tobacco, Arabidopsis, flax, soybean and many others have been successfully transformed with *nptII*. Also lettuce has been transformed with this gene by several groups [8,9,10,11]. This gene codes for aminoglycoside 3′-phosphotransferase, which inactivates aminoglycoside antibiotics such as kanamycin, neomycin and gentamycin by phosphorylation [12], 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 specie, explant type, developmental stage and tissue culture conditions [13]. 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 [14]. Another simple scheme for evaluation of transgenic plants was developed for testing sorghum plants containing the *bar* gene [15] 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 was 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.

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 [16] and *nptII* gene. Genetic transformation of lettuce was performed following the protocol previously established by our group. Young leaves were transformed by co-cultivation with the LBA4404 *Agrobacterium tumefaciens* strain

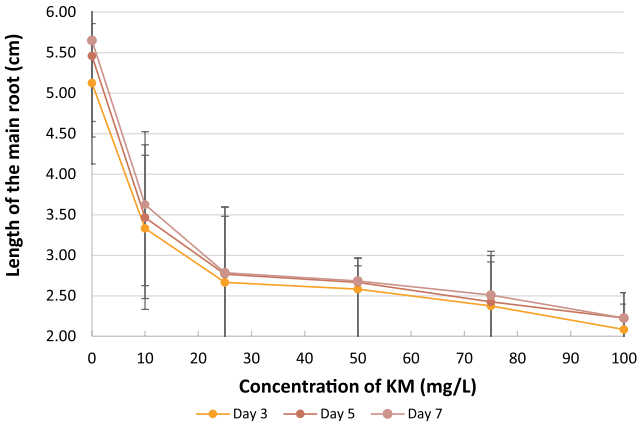

Fig. 2. Length of the main root of non-transgenic seedlings germinated at different concentrations of Km. Average length of the main root of non-transgenic seedlings germinated in 1/2 MS supplemented with 0, 10, 25, 50, 75 and 100 mg/L of Km at 3, 5 and 7 d. Bars indicate standard errors.

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