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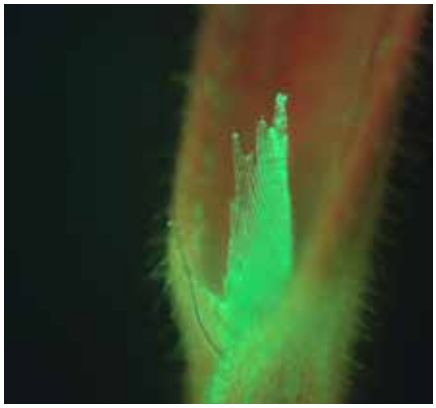

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DETERMINATION OF SURVIVAL MOTOR NEURON (SMN) GENES COPY NUMBERS IN A SAMPLE OF HEALTHY POPULATION FROM SOUTHERN SPAIN

DETERMINACIÓN DEL NÚMERO DE COPIAS DE GENES DE SUPERVIVENCIA DE LA NEURONA MOTORA (SMN) EN UNA MUESTRA DE UNA POBLACIÓN SANA DEL SUR DE ESPAÑA

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ABSTRACT

Copy number analysis of the *SMN* (Survival Motor Neuron) genes in healthy individuals with no history of spinal muscular atrophy (SMA) is important to assess carrier frequency, the frequency of patients with two copies of *SMN1* on one chromosome (a factor that could lead to a false-negative result when testing for SMA carriers) and the mechanisms responsible for these chromosomes with two copies of *SMN1*. We retrospectively analyzed the copy number of *SMN1* and *SMN2* genes detected in blood samples of 119 gamete donors by Multiplex Ligation-Dependent Probe Amplification (MLPA) assay during the last two years. The number of donors with a heterozygous deletion of exon 7 of *SMN1* was 1 in 59 samples (1.7 %). It was estimated that 5.4 % of the studied alleles presented two *SMN1* copies. The percentage of individuals with one or zero copies of *SMN2* was not statistically different among individuals with two copies of *SMN1* (48.1 %) and those with three or four *SMN1* copies (63.6 %) (p= 0.327). The frequency of individuals with one copy of *SMN1* is low and consistent with previously reported data; therefore, universal screening is not cost-effective, although it could be so in gamete donors. Our results are not in agreement with the hypothesis that the occurrence of gene conversion from *SMN2* to *SMN1* results in two *SMN1* copies on one chromosome.

Key words: SMN genes, SMA, carrier, gene conversion.

RESUMEN

El análisis del número de copias de los genes *SMN* (de sus siglas en inglés *Survival Motor Neuron*)en población sana sin historia de atrofia muscular espinal (AME) es importante para establecer la frecuencia de portadores, la frecuencia de pacientes con dos copias de *SMN1* en un cromosoma (un factor que puede dar lugar a falsos negativos cuando se realizan estudios de portadores) y los mecanismos responsables de estos cromosomas con dos copias de *SMN1*. Hemos analizado retrospectivamente el número de copias de los genes *SMN1* y *SMN2* detectadas por MLPA (amplificación de sondas dependiente de ligandos múltiples, de sus siglas en inglés *Multiplex Ligation-dependent Probe Amplification*) en muestras de sangre de 119 donantes de gametos durante los dos últimos años. El número de donantes con una deleción en heterocigosis del exón 7 del *SMN1* fue de uno cada 59 muestras (1,7 %). La estimación del porcentaje de alelos estudiados con dos copias de SMN1 fue de 5,4 % y el porcentaje de individuos con una o ninguna copia de SMN2 no fue estadísticamente significativo entre los individuos con dos copias de *SMN1* (48,1 %) y aquellos con tres o cuatro copias (63,6 %) (p=0,327). La frecuencia de individuos con una copia de *SMN1* es baja y consistente con datos anteriores publicados, por lo que el tamizado universal no es efectivo en términos de costo, aunque podría serlo en donantes de gametos. Nuestros resultados no están de acuerdo con la hipótesis de que la conversión de *SMN2* a *SMN1* da lugar a cromosomas con dos copias de *SMN1*.

Palabras clave: genes SMN, AME, portador, conversión génica.

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INTRODUCTION

Spinal muscular atrophy (SMA) is the second most common lethal autosomal recessive disorder after cystic fibrosis in the western hemisphere (Ogino *et al.*, 2002). The carrier frequency is between 1/40 and 1/60 in diverse populations, affecting 1 in 10,000 live births (Cusin *et al.*, 2003; Feldkötter *et al.*, 2002. This disorder is characterized by the degeneration of the lower motor neurons of the spinal cord, leading to symmetrical muscle weakness, atrophy and, in the majority of cases, premature death.

SMA is caused by mutation in the survival motor neuron 1 (*SMN1*) gene (Lefebvre *et al.*, 1995). A 96 % of the SMA patients present a homozygous deletion of at least *SMN1* exon 7. The remaining 4 % of cases are caused by a variety of molecular lesions, including point mutations, small deletions and small insertions (Wirth, 2000; Monani *et al.*, 1999).

Survival motor neuron 2 (*SMN2*) gene is highly similar to *SMN1*. It differs from *SMN1* in only five nucleotides: two in exonic positions (exon 7 and exon 8) and three in intronic positions (one in intron 6 and two in intron 7) (Wirth *et al.*, 1999). The nucleotide change in exon 7 of *SMN2* alters an exonic splicing enhancer. As a result, most SMN2 transcripts lack exon 7 and are not functional, being more than 90 % of the functional SMN protein due only to the action of *SMN1* gene (Lorson *et al.*, 1999). Therefore, it is *SMN1* copy number, and not *SMN2*, which determines the patient status. *SMN2* acts only as a disease-modifying gene: the more SMN2 copies a patient has, the milder the SMA phenotype is.

The copy number of *SMN1* ranges from zero to four. Two, three or four copies may be detected in healthy subjects. Nevertheless, in the vast? majority of SMA carriers only one copy of *SMN1* is identified, although some SMA carriers are characterized by the presence of two *SMN1* copies on only one chromosome of the pair. No copies of the *SMN1* exon 7 are present in most SMA patients.

Most screening methods for carriers, including the multiplex ligation-dependent probe amplification (MLPA) assay, typically perform a quantitative analysis of *SMN1* exon 7 copy number. These methods do not allow the detection of either point mutations, small deletions or small insertions. Moreover, it is not possible to distinguish carriers with two copies on one chromosome from "wild type" patients, with one copy on each chromosome of the pair.

Copy number analysis of the *SMN1* gene in healthy individuals with no history of SMA is important to assess carrier frequency in each population and to be able to recommend universal screening. Hendrickson *et al.* (2009) reported that the frequency of SMA carrier is different among different ethnic groups. Moreover, analysis of the SMA locus may result useful to evaluate the frequency of patients with two copies of *SMN1* on one chromosome, an important factor that can modify the rate of being SMA carrier in a population. An assessment of *SMN2* copy number may help to explain the presence of two copies of *SMN1* on one chromosome.

METHODS

In the study reported herein, we retrospectively analyzed the copy number of *SMN1* and *SMN2* genes that had been detected in blood samples of 119 gamete donors by MLPA assay during 2012 and 2013. All subjects studied were born in the Andalusia region and did not have a family history of SMA.

MLPA was performed using Kit P060 version B2 (MRC Holland, Netherlands). This MLPA probemix detects copy number changes of exon 7 and 8 of the *SMN1* and *SMN2* genes. Analysis of PCR products was done in a 3130ABI sequencer (Applied Biosystems, USA). Data was further studied with Genemapper® software v4.1.

RESULTS

The number and the percentage of different *SMN1*/*SMN2* genotypes observed in our study are shown in Table 1, and the frequencies of the *SMN1* alleles are listed in Table 2.

Two *SMN1* copies were the more prevalent genotype (89.1 %), whereas the frequency of individuals with only one *SMN1* copy (and, therefore, carriers of SMA) was low, 1 in 59 samples (1.7 %).

Nine and two of the 119 patients studied presented three and four copies of *SMN1*, respectively. Hence, 13 alleles of the 238 studied was estimated to be two copy-alleles (5.4 %). Consequently, it is possible that there were more SMA carriers among those individuals in which two copies of *SMN1* were detected.

Previously published data have shown that increased *SMN1* copy number is associated with decreased *SMN2*

copy number due probably to gene conversion from *SMN2* to *SMN1* (Ogino *et al.*, 2003). Nevertheless, in our study the percentage of individuals with one or zero copies of *SMN2* was statistically non-significant among individuals with two copies of *SMN1* (48.1 %) and those with three or four *SMN1* copies (63.6 %) (p=0.387, χ^2 test).

Table 1. *SMN1* and *SMN2* copy numbers in a sample of 119 gamete donors from southern Spain

Copy relations SMN1: SMN2	N° of subjects	Status
1:3	2	SMA carrier
2:0	9	non-carrier
2:1	42	non-carrier
2:2	52	non-carrier
2:3	3	non-carrier
3:0	1	non-carrier
3:1	4	non-carrier
3:2	4	non-carrier
4:0	1	non-carrier
4:1	1	non-carrier

Table 2. *SMN1* copies observed in the 119 gamete donors studied

Number of allele copies	Number of donors (%)
1	2 (1.7)
2	106 (89.1)
3	9 (7.5)
4	2 (1.7)

DISCUSSION

Using *SMN* gene dosage analysis, we studied *SMN1* and *SMN2* copy numbers in a sample of a healthy population from Andalusia. To our knowledge, it is the first study of these characteristics performed in the above mentioned area.

The frequency of individuals with a heterozygous deletion of exon 7 of *SMN1* gene in our cohort is consistent with the prevalence of SMA carriers in previously reported data. Nevertheless, there may be a few more carriers in the sample studied because the MLPA assay does not allow both the detection of some types of uncommon mutations and the identification of carriers with two *SMN1* copies on one chromosome and zero in the other of the pair. The number of alleles with two, three and four copies of *SMN* genes also agrees with published data from other authors in other populations? (Ogino *et al.*, 2004; Anhuf *et al.*, 2003).

The fact that three or more copies of *SMN1* were detected in some individuals, indicates the presence of alleles with two copies of *SMN1* on one chromosome. This is a fundamental factor that could lead to a false-negative result when testing for SMA carriers and two copies are identified. Calculations for estimating the probability of being an SMA carrier in an individual without a family history of SMA and two copies of *SMN1*, take into consideration the estimated frequency of alleles with two copies of *SMN1*. In Caucasian populations and for an estimated frequency of two *SMN1* copy similar to detected by us (5.4 %), the adjusted risk is approximately 1:632 (Smith *et al.*, 2007). Rare mutations, undetectable by MLPA, are also taken into account for these calculations.

Therefore, it is important to determine how many *SMN1* alleles present more than two copies in order to obtain an estimated frequency of 2-copy alleles in each population and to calculate the probability of being an SMA carrier even though two *SMN1* copies had been identified.

The mechanisms responsible for the presence of two copies of *SMN*1 on one chromosome 5 remain unknown. Ogino *et al.* (2003) studied 13 individuals with three or four copies of *SMN1*, and speculated that the occurrence of gene conversion from *SMN2* to *SMN1* results in two *SMN1* copies on one chromosome. We evaluated a similar number of this type of patients. Our results are in disagreement with Ogino´s et al. (2003) observation because if this were so, increased *SMN1* copy number would be associated with decreased *SMN2* copy number. In the sample studied, copies of *SMN2* are similar among individuals with two copies of *SMN1* and those with three or four *SMN1* copies. Additional investigations with a higher number of patients with three and four copies of the *SMN1* gene are necessary to ascertain if the hypothesis of gene conversion from *SMN2* to *SMN1* should be accepted.

Although guidelines from the American College of Medical Genetics (ACMG) published in 2008 recommend universal carrier screening for SMA, this screening is not cost-effective, as has yet been studied elsewhere (Little *et al.*, 2010). However, in gamete donors, and given that these gametes may be used by several recipients, there is an increased risk of autosomal recessive disease in the offspring of multiple pairings over that of a single couple. Hence, it is reasonable to perform screening of potential gamete donors for common and severe disorders such as SMA.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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IDENTIFICATION OF FRESHWATER HYDROPHYTES FOR GENOTOXICITY ASSESSMENT OF AQUATIC POLLUTANTS

IDENTIFICACIÓN DE HIDRÓFITAS DULCEACUÍCOLAS PARA ESTUDIOS DE GENOTOXICIDAD DE CONTAMINANTES ACUÁTICOS

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ABSTRACT

For exploring the potential use of hydrophytes in genotoxicity bioassays for aquatic contaminants, as a complement or replacement of the traditional and standardized use of terrestrial macrophytes (*Allium cepa* (L.), *Pisum sativum* (L.), *Tradescantia* sp. (L.), among others), a preliminary study was carried out in an attempt to select at least one wild wetland taxonomic species that had to meet the following requirements: to have a low basic chromosome number and a high mitotic index (MI), and to be easily propagated in the laboratory. Three field vegetation surveys were carried out in La Brava Lake (Buenos Aires province, Argentina). Seeds of the most representative species were collected and placed in Petri dishes for germination. Cytogenetic studies were, then, performed in root tips. Two of the species, *Bidens laevis* (L.) and *Solanum chenopodioides* (Lam.) fulfilled the requirements for their use in the analysis of genotoxicity of aquatic contaminants.

Key words: native aquatic macrophytes, basic chromosome number, mitotic index, cytogenetic bioassays, genotoxicity.

RESUMEN

Con el objeto de explorar el uso de hidrófitas en ensayos de genotoxicidad de contaminantes acuáticos, como complemento o reemplazo del uso tradicional y estandarizado de macrófitas terrestres (*Allium cepa* (L.), *Pisum sativum* (L.), *Tradescantia* sp., entre otras), se llevó a cabo un estudio preliminar en un intento por seleccionar al menos una especie taxonómica silvestre dulceacuícola que debe cumplir los siguientes requisitos: poseer número cromosómico básico bajo, índice mitótico (IM) elevado y ser fácil de propagar en laboratorio. Para tal fin, se realizaron tres campañas de relevamiento de vegetación en la Laguna La Brava (provincia de Buenos Aires, Argentina). Se recolectaron semillas de las especies más representativas, y se dispusieron en placas de Petri para su germinación. Luego se realizaron estudios citogenéticos en ápices radicales. Las especies taxonómicas *Bidens laevis* (L.) y *Solanum chenopodioides* (Lam.) cumplieron los requisitos para su uso en el análisis de genotoxicidad de contaminantes acuáticos.

Palabras clave: macrófitas acuáticas nativas, número cromosómico básico, índice mitótico, bioensayos citogenéticos, genotoxicidad.

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INTRODUCCIÓN

El primer intento de evaluación de ensayos de mutagenicidad con plantas fue llevado a cabo por la Agencia de Protección Ambiental de Estados Unidos de América (USEPA) con el programa Gene-Tox, publicado en 1982 (Constantin y Owens, 1982). Este programa tenía como fin la evaluación del estado de los bioensayos de genética toxicológica en microorganismos, animales y plantas. Los resultados mostraron que los ensayos en plantas tenían alta sensibilidad para predecir la actividad mutagénica de compuestos químicos, es decir que se observaban pocos falsos negativos, por lo que resultaban ser sumamente confiables. En general el análisis de los datos mostró una alta concordancia entre los ensayos en plantas y mamíferos (Ennever *et al.*, 1988). Sin embargo, algunas sustancias químicas que daban respuestas negativas en animales tenían resultados positivos en plantas, como es el caso de la hidrazida maleica (HM) (Gichner *et al.*, 1982; Alvarez Moya *et al.*, 2001; Rank *et al.*, 2002).

En 1984 se inició un estudio colaborativo con plantas superiores, dentro del *International Program on Chemical Safety* (IPCS) bajo el auspicio del Programa de las Naciones Unidas para el Desarrollo (PNUD) y la Organización Mundial de la Salud (OMS). En dicho estudio, se proponía la evaluación de ensayos en plantas a corto plazo para la detección de sustancias potencialmente mutagénicas y carcinogénicas, y la posibilidad de aplicar estos ensayos, en particular en países en desarrollo. Entre otros utilizados, se encuentran los bioensayos de pelos estaminales en *Tradescantia palludosa* (E.S. Anderson & Woodson) para detectar mutaciones génicas, aberraciones cromosómicas en ápices de raíz en *Vicia faba* (L.) y micronúcleos (MN) en *T. palludosa*, para evaluar los químicos hidrazida maleica (HM), N-nitroso-N-metilurea (MNU), azida sódica (NaN_3), azidoglicerol (AG) y etil metanosulfonato (EMS).

Se concluyó que los ensayos con plantas son sistemas eficientes y confiables para un monitoreo rápido de mutagenicidad y clastogenicidad de compuestos químicos (Shandu *et al.*, 1994). Si bien solamente fueron validados tres bioensayos a través del programa internacional IPCS, cualquier sistema con plantas superiores que sea validado y demuestre ser eficiente para el monitoreo *in situ* o para ensayos de laboratorio, podría ser incluido en estudios futuros dentro de este marco (Ma, 1998).

Particularmente, la Angiosperma terrestre *Allium cepa* (L.) fue introducida en 1938 como sistema de prueba para determinar los efectos de la colquicina (Maluszynska y Juchimiuk, 2005). Posteriormente se utilizó dicha especie para analizar la genotoxicidad de compuestos químicos mediante el ensayo de aberraciones cromosómicas, restricciones en el crecimiento de raíces y concentraciones efectivas (EC) de diferentes tóxicos facilitándose así la comparación con otros ensayos de toxicidad (Fiskesjö, 1997). Este ensayo se puede aplicar para la evaluación de aguas provenientes de fuentes naturales (ríos, lagos, lagunas), de bebida, de efluentes domésticos e industriales y de químicos solubles e insolubles en agua (Wulff y Andrioli, 2006), y presenta la ventaja de ser de rápida aplicación, económico y fácil de llevar a cabo. Sin embargo, en la literatura se reconoce la necesidad de hallar nuevas especies sensibles para realizar bioensayos de toxicidad (Vervliet-Scheebaum *et al.*, 2006), entre los que pueden incluirse los de genotoxicidad como el ensayo de aberraciones cromosómicas en anafase-telofase (EACAT).

En la mayoría de los bioensayos previamente mencionados se utilizan plantas terrestres bioindicadoras (Rank, 2003), que resultan ser una herramienta de uso corriente en la evaluación de efectos genotóxicos, pero los resultados son relevantes sólo cuando se aplican en ambientes terrestres. Algunos autores, en contraposición a este hecho, reconocen la gran utilidad de los bioensayos con plantas estándares terrestres para la detección de genotoxinas en el ambiente acuático (Majer *et al.*, 2005). Sin embargo, el uso de plantas terrestres no revela el impacto de compuestos genotóxicos sobre las poblaciones de plantas acuáticas crónicamente expuestas en su hábitat natural, y es por ello que los estudios pueden llevar a resultados alejados de la realidad o falsos positivos (Latuzka *et al.*, 2003). Estos problemas pueden ser solucionados si se utilizan especies indicadoras que son parte de la flora natural del lugar de monitoreo, porque los datos que se obtengan serán más ajustados a la realidad del ambiente que se analiza. Por esta razón, el uso de hidrófitas como bioindicadoras de efectos genotóxicos constituye una herramienta irremplazable para el monitoreo y la conservación de ecosistemas litorales (Ferrat *et al.*, 2003). Así, el objetivo general del presente trabajo fue seleccionar especies silvestres de ecosistemas dulceacuícolas que reunieran características apropiadas

para su utilización en bioensayos de genotoxicidad de tóxicos presentes en el medio acuático, para lo cual deben cumplir con los siguientes requisitos: ser fáciles de propagar en laboratorio, y poseer bajo número cromosómico básico y elevado índice mitótico (IM).

MATERIALES Y MÉTODOS

Área de muestreo y material biológico

El relevamiento de especies fue realizado entre los meses de abril y mayo de 2004, y consistió de tres salidas de campo en las que se muestrearon macrófitas acuáticas y palustres en la Laguna La Brava, provincia de Buenos Aires (37° 53' Latitud Sur, 57° 59' Longitud Oeste). Las estaciones de muestreo se ubicaron en el tributario de dicha laguna, el arroyo El Peligro, y en su emisario, el arroyo Tajamar. La condición taxonómica de los ejemplares recolectados se determinó mediante el uso de las claves dicotómicas de Morton (1976), Petetín *et al*. (1977), Cabrera y Zardini (1978), Orchard (1981) y Boelcke y Vizinis (1987). La caracterización de las especies se realizó utilizando el Catálogo de Plantas Vasculares del Conosur (CPVC 2013).

De las especies seleccionadas acorde a su representatividad en el lugar, se recolectaron flores maduras en bolsas de papel, que se etiquetaron, y se aislaron las semillas. Una vez secas, las semillas se lavaron con una mezcla de agua destilada: hipoclorito de sodio 1:1 v/v, durante 5 min, con agitación constante a 20° C, usando un agitador magnético para lograr una correcta desinfección superficial. Para las especies acuáticas *Myriophyllum quitense* (*Kunth*), *Ludwigia peploides* (H.B.K.) e *Hydrocotile ranunculoides* (L.) se recolectaron ejemplares que fueron colocados en acuarios de vidrio, debido a que su modo de reproducción es principalmente por propagación vegetativa. Por ello, para estas especies no se calculó el poder germinativo de las semillas.

Cálculo del porcentaje de germinación y capacidad de propagación en laboratorio

Se acondicionaron semillas (n= 21) de cada especie en cajas de Petri, con algodón húmedo y papel de filtro para su germinación y posterior obtención de plántulas, en cámara de crecimiento a 22° C, y en oscuridad. El número y distribución de las semillas fue uniforme en cada caja con el propósito de estandarizar las condiciones de crecimiento de las plántulas. Luego de 15 días se cuantificó el número total de semillas germinadas por especie, evaluando la emergencia de cotiledones y radícula, y el poder germinativo se expresó como porcentaje del total de semillas evaluadas.

Cálculo del índice mitótico y del número cromosómico

Las semillas germinadas fueron transferidas a macetas que contenían como sustrato una mezcla de humus: turba 70:30 p/p aproximadamente, y las plántulas derivadas se cultivaron en invernáculo. Para el cálculo del índice mitótico, se seleccionaron entre cinco y ocho plántulas de cada especie, de las que se cosecharon raíces de no más de 1 cm de longitud, las cuales se transfirieron a una solución mezcla de etanol: ácido acético glacial 3:1 v/v durante 24 h, para la fijación del tejido. Luego, las raíces fijadas se transfirieron a etanol 70 % para su conservación en heladera a 4° C hasta el procesamiento. La hidrólisis se realizó en una solución de HCl 1N durante 10 min a 60° C en baño termostático, luego del lavado de las raíces con agua destilada. Las raíces hidrolizadas se enjuagaron dos veces con agua destilada y se tiñeron con solución de Feulgen (fucsina leucobásica) durante 2 h en oscuridad. Posteriormente, se colocaron en un portaobjetos con una gota de solución de carmín 1-2 % en ácido acético 45 %. El extremo apical, localizado mediante observación bajo lupa, fue separado del resto del material con un bisturí y desmenuzado con una varilla de vidrio. Por último se realizó el aplastamiento de la muestra con cubreobjetos sobre portaobjetos (técnica de "squash"). Los preparados realizados se observaron mediante microscopía óptica, con un microscopio Olympus BH–2. El índice mitótico (IM) fue calculado como el número de células en división mitótica (estadios de profase, metafase, anafase y/o telofase) cada 1.000 células observadas y fue expresado en porcentaje.

Para la determinación del número cromosómico se seleccionaron entre cinco y ocho plántulas de cada especie y se cosecharon raíces de no más de 1 cm de longitud. Dichas raíces se transfirieron a una solución de 8-hidroxiquinoleína durante 2-4 h, continuándose luego con el mismo procedimiento descripto previamente para el cálculo del índice mitótico. El número cromosómico fue determinado en al menos cinco campos microscópicos en c-mitosis.

RESULTADOS

Entre las especies taxonómicas más frecuentes en las orillas de la Laguna La Brava se encontraron *Cleome titubans* (Speg.), *Typha latifolia* (L.), *Ludwigia peploides (H.B.K.), Solanum chenopodioides* (Lam.), *Myriophyllum quitense* (Kunth), *Schoenoplectus californicus* (C.A. Meyer), *Bidens laevis* (L.) *Hydrocotile ranunculoides* (L.), *Polygonum persicaria* (L.)..

En la Tabla 1 se presenta la clasificación taxonómica, hábito de crecimiento y status para cada una de las especies seleccionadas. Las semillas de *T. latifolia*, *S. californicus* presentaron poder germinativo menor a 2 %, hecho que dificultó la obtención de plántulas para realizar el recuento cromosómico y los bioensayos. Sin embargo, para ambas especies se observó que las células eran poliploides; aunque no se pudo establecer el número cromosómico en forma precisa, se estimó que el mismo es mayor a 50. Del mismo modo, las especies *L. peploides* y *H. ranunculoides* resultaron ser poliploides, con números cromosómicos somáticos mayores a 50 (Tabla 2, Figura 1), teniendo en cuenta que poseen especies estrechamente emparentadas con n=x=8 y n=x= 12 (Raven y Tai, 1979; Murray *et al.*, 2012).

El número cromosómico somático de la macrófita acuática *M. quitense* fue 2n= 14, pero esta especie no presentó un IM apropiado para ser utilizada en el EACAT, ni un número suficiente de células interfásicas para el recuento de micronúcleos (MN). Las especies *B. laevis*, *C. titubans*, *S. chenopodioides* y *P. persicaria* resultaron de fácil propagación, y con un número cromosómico apropiado para realizar los bioensayos (2n= 24, 2n= 14, 2n= 22 y 24, y 2n= 22, respectivamente) (Figura 1). *Cleome titubans* y *P. persicaria* presentaron IM inferiores a 1 % mientras que *S. chenopodioides* y *B. laevis* mostraron valores entre 3-12 % (Tabla 2).

Tabla 1. Clasificación taxonómica, hábito de crecimiento y status de las hidrófitas dulceacuícolas estudiadas

Especie taxonómica	Nombre vulgar	Familia	Hábito	Status
Cleome titubans (Speg.)	Flor de araña chica	Capparaceae	Hierba	Nativa
Typha latifolia (L.)	Totora	Typhaceae	Hierba perenne	Cosmopolita
Ludwigia peploides (H.B.K.)	Falsa verdolaga	Onagraceae	Hierba perenne	Nativa
Solanum chenopodioides (Lam.)	Hierba mora	Solanaceae	Arbusto perenne	Nativa
Myriophyllum quitense (Kunth)	Gambarrusa	Haloragaceae	Hierba acuática perenne	Nativa
Schoenoplectus californicus (C.A. Meyer)	Junco	Cyperaceae	Hierba acuática perenne	Nativa
Bidens laevis (L.)	Amor seco	Asteraceae	Hierba perenne	Nativa
Hydrocotyle ranunculoides (L.)	Redondita de agua	Apiaceae	Hierba perenne	Nativa
Polygonum persicaria (L.)	Moco de pavo	Polygonaceae	Hierba anual	Adventicia

Tabla 2. Características citológicas de las hidrófitas dulceacuícolas estudiadas

Especie taxonómica	Poder germinativo de semillas	Mortalidad de plántulas	Número Cromosómico somático	Índice mitótico (%)	Número Cromosómico Básico descripto en Género (G) y/o Especie (E)*
Cleome titubans	≥ 50	Escasa	2n= 14	< 1 %	*x*= 9, 10, 11, 12, 13, 14 (G)
Typha latifolia	≤ 2	n.a.	n.a.	n.a.	*x* = 15 (G); 2n= 30 (E)
Ludwigia peploides	n.a.	n.a.	> 50	n.a.	Jussieua** *x*= 8; *J. repens* 2n= 16
Solanum chenopodioides	≥ 70	Nula	2n= 22 y 24	3-12	*x*= 12 (G)
Myriophyllum quitense	n.a.	Nula	2n= 14	< 1	*x*= 7 (G)
Schoenoplectus californicus	≤ 2	n.a.	> 50	n.a.	*x*= 20, 21 (G)
Bidens laevis	≥ 70	Nula	2n= 24	4-12	*x*= 12 (G)
Hydrocotyle ranunculoides	n.a.	n.a.	> 50	n.a.	*x*= 8, 9, 11 (Murray *et al.*, 2012)
Polygonum persicaria	≥ 70	Nula	2n= 22	< 1	*x*= 11 (G); 2n = 4x = 44 (E)

n.a.: no analizado. * tomados de Darlington y Wylie (1961), excepto para Hydrocotyle ranunculoides. **Actualmente género Ludwigia.

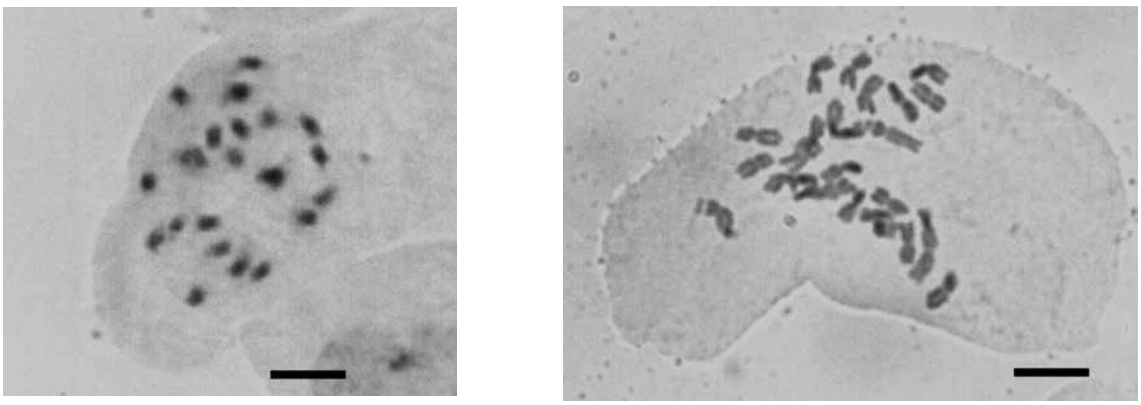

Figura 1. Cromosomas en las hidrófitas *Bidens laevis* y *Solanum chenopodioides.* Tamaño de la barra: 5 micrones.

DISCUSIÓN

Para estudios de genotoxicidad *in situ* se ha propuesto transportar la especie terrestre *Vicia faba* a sitios de contaminación potencial, por lo que se la haría crecer en ambientes que no son naturales para la especie (Grant y Owens, 2001). Por el contrario, el uso de hidrófitas en el biomonitoreo de contaminantes de ecosistemas acuáticos proporcionaría datos más ajustados a la realidad que los que puedan obtenerse con las especies modelo terrestres usadas actualmente. Por eso, se plantea la necesidad de identificar especies vegetales vinculadas al medio acuático que permitan revelar el verdadero impacto de compuestos genotóxicos sobre las poblaciones autóctonas. Además, hay que tener en cuenta que las poblaciones de especies introducidas pueden estar adaptadas a tóxicos ambientales por lo que los resultados que se obtienen pueden estar alejados de la realidad o ser falsos negativos (Lazutka *et al*., 2003). En el presente estudio se evaluaron características citológicas, hábito de crecimiento y clasificación taxonómica de especies pertenecientes a ecosistemas acuáticos lagunares, identificándose a *B. laevis* y *S. chenopodioides* como aquellas que cumplen con los requisitos para su utilización en la evaluación de genotoxicidad de contaminantes acuáticos.

El género *Bidens* es muy amplio, ya que contiene alrededor de 240 especies taxonómicas. En Argentina, crecen espontáneamente diez de dichas especies, de las cuales tres están ampliamente distribuidas en la provincia de Buenos Aires, *B. laevis*, *B. pilosa* y *B. subalternans* (Cabrera y Zardini, 1978). *B. laevis* es la única que habita en ecosistemas acuáticos, siendo nativa y representativa de los ambientes lagunares pampásicos. Presenta una amplia distribución en toda América, desde Estados Unidos de América, México y Colombia hasta Chile, Uruguay y Argentina (Lahitte y Hurrell, 1997). En Argentina, se halla en las provincias de Buenos Aires, Chaco, Córdoba, Corrientes, Entre Ríos, Formosa, Jujuy, Misiones, Salta, Santa Fe y San Juan (Cabrera *et al*., 2000; Sáenz y Tombesi, 2003), creciendo comúnmente en suelos pantanosos, orillas de ríos y arroyos, en esteros y sobre embalsados flotantes, hasta los 500 m sobre el nivel del mar siendo, asimismo, característica de los pajonales de la ribera del Plata (Cabrera y Zardini, 1978; Sáenz y Tombesi, 2003). Es una macrófita herbácea perenne que, similarmente a muchas otras plantas palustres o helófitas, durante los meses de invierno conserva sólo un tallo subterráneo dentro del sedimento y se hace conspicua a partir de la primavera, floreciendo en el sudeste bonaerense entre enero y mayo, y atravesando luego un periodo de disecación de la parte emergente en junio-agosto.

Por su parte, el género *Solanum* es también muy amplio, e incluye 243 especies taxonómicas en la Argentina (CPVC, 2013), tanto terrestres como palustres. Dos de ellas, *S. tuberosum* (papa) y *S. lycopersicum* (tomate), son de gran importancia para la alimentación. La especie *S. chenopodioides* (sinónimo de *S. sublobatum* Willd) se encuentra en las provincias de Buenos Aires, Córdoba, Entre Ríos, Misiones, Río Negro, Santa Fe y Neuquén en Argentina, así como en Brasil, Uruguay y Paraguay (CPVC, 2013).

Los ambientes pampásicos donde habitan *B. laevis* y *S. chenopodioides* son regiones de intensa actividad agrícola, motivo por el cual la llegada de agroquímicos a los cuerpos de agua superficiales por fenómenos de escorrentía, lixiviación, transporte atmosféricos, entre otros, es un factor crucial que puede determinar la dinámica poblacional de todo el ecosistema acuático y, en particular, la bioacumulación de estos compuestos en las macrófitas con los consiguientes efectos adversos.

La evaluación de aberraciones cromosómicas consume tiempo pero es facilitada por el uso de especies indicadoras que poseen número bajo de cromosomas de tamaño grande, siendo las especies *Tradescantia* sp., *Crepis capillaris, Vicia faba* y *Allium cepa* las que cumplen con este criterio (Uhl *et al*., 2003). La especie *B. laevis* ya ha sido utilizada por nuestro grupo de trabajo en bioensayos de genotoxicidad, habiéndose demostrado que es una especie de sensibilidad elevada en comparación con *Allium cepa* (cebolla) cuando fue expuesta a reconocidos mutágenos como metil metanosulfonato (MMS), ENU y el herbicida HM (Pérez *et al*., 2011). Además, esta especie mostró un incremento en la frecuencia de ACAT y cambios en enzimas de estrés oxidativo al ser expuesta al insecticida organoclorado endosulfán (Pérez *et al*., 2008; 2011; 2013).

Entre las especies más abundantes en biomasa en las lagunas pampásicas, y por esto las más representativas, están *S. californicus* y *M. quitense* (Lahitte y Hurrell, 1997). Sin embargo, y con respecto a las características analizadas en este trabajo, ambas especies resultaron no ser aptas para ensayos de genotoxicidad debido a que la primera es poliploide y las semillas de la segunda presentan un poder germinativo extremadamente bajo; asimismo, el número de células interfásicas es insuficiente para el recuento de

MN posiblemente debido a la presencia de abundante aerénquima en la raíz, por lo que fueron descartadas como posibles especies modelo. Por otra parte, se debe resaltar que en el EACAT utilizando la especie terrestre *Allium cepa* para la evaluación de diversos agentes mutagénicos es frecuente observar variaciones amplias en los IM de las muestras de los tratamientos controles (Rank, 2003). Por este motivo, se cuestiona si este índice debe ser utilizado como una medida cuantitativa de la genotoxicidad de los compuestos cuyo efecto se desea caracterizar. Varios autores consideran que sólo hay que tenerlo en cuenta para determinar si en las muestras se encontrará la cantidad mínima de células en división necesarias para llevar a cabo el recuento de células en anafase-telofase en el EACAT. Los IM obtenidos en las raíces de plántulas de *B. laevis* y *S. chenopodioides* se corresponden con los valores recomendados por Rank (2003) (>1 %) para llevar a cabo el EACAT, cumpliendo así con uno de los requisitos para que estas especies puedan ser utilizadas en análisis de genotoxicidad. Otra característica importante que poseen ambas especies es el número cromosómico relativamente bajo, lo que es recomendable (Majer *et al.*, 2005), como en *Tradescantia paludosa* (2n= 24) (Sobham *et al.*, 1991).

La identificación de especies acuáticas y/o palustres potencialmente útiles para realizar bioensayos de genotoxicidad y/o biomonitoreo *in situ* en ambientes lagunares es de gran interés para el avance del conocimiento en ecogenotoxicidad, es decir, en la susceptibilidad genotóxica de la flora silvestre en vistas al desarrollo de estrategias para el mantenimiento de la biodiversidad de ciertos biomas. Por otro lado, estos estudios cobran relevancia por su posible uso en ambientes sustentables, disminuyendo el riesgo de la alteración del equilibrio ante la exposición a insecticidas propios de las economías agrícolas de ciertas zonas de nuestro país (Mudry com. pers., 2009). En conclusión, entre las especies taxonómicas nativas evaluadas, *B. laevis* y *S. chenopodioides* resultaron ser las más adecuadas para su utilización en estudios de genotoxicidad, tanto para el biomonitoreo *in situ* como para bioensayos.

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TRANSFORMATION OF AN ARGENTINE SPRING WHEAT GENOTYPE: OPTIMIZATION OF THE PROTOCOLS FOR PARTICLE BOMBARDMENT OF EXCISED IMMATURE EMBRYOS AND RAPID ISOLATION OF TRANSGENIC PLANTS

TRANSFORMACIÓN DE UN GENOTIPO ARGENTINO DE TRIGO PRIMAVERAL: OPTIMIZACIÓN DE LOS PROTOCOLOS PARA EL BOMBARDEO DE PARTÍCULAS DE EMBRIONES INMADUROS EXTRAÍDOS Y AISLAMIENTO RÁPIDO DE PLANTAS TRANSGÉNICAS

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ABSTRACT

Klein Brujo was found to be a promising Argentine spring wheat genotype for transformation studies. In the present work we optimized the biolistic transformation of embryogenic scutellar calli from this genotype. We first identified scutellar callus induction media (SCIM) most promising for *in vitro* embryogenic plant regeneration with *Klein Brujo*. We then co-bombarded embryos of *Klein Brujo* and, for comparison, of *Bobwhite*, a highly transformable wheat line, on these media with 1 µm gold particles coated with two plasmids. One of these contained the marker gene *gfp* (linked to the *CaMV35S*-promoter) and the selection gene *bar* (resistance to phosphinothricin: PPT, linked to the maize *Ubi1*-promoter), whereas the other contained *ipt* (encoding isopentenyltransferase) as a candidate gene under the control of the *HvS40*- or *SAG12*- promoter. Transformation efficiencies of up to 16.4% with *Klein Brujo* and 6% with *Bobwhite* were obtained with embryos pre-cultured on SCIM for 96 h and subjected to pre- and post-bombardment osmotic treatment for 4-5 and 16 h, respectively. Transgenic calli and plants regenerating *in vitro* were identified by screening for GFP expression and PPT resistance. One hundred and three transgenic lines of *Klein Brujo* - far more than of *Bobwhite* (12 lines) - were established on soil, often within only nine weeks. The frequency of co-transformation of *gfp* and/or *bar* and *ipt* exceeded 97% for both genotypes, and the three genes were shown to co-segregate with selected individuals. The *ipt*-gene was structurally stable up to the T3 generation, whereas *gfp* and *bar* were susceptible to silencing.

Key words: Particle bombardment parameters; co-transformation; green fluorescent protein; phosphinothricin resistance; transgene segregation.

RESUMEN

Klein Brujo es un genotipo argentino de trigo de primavera prometedor para estudios de transformación. En el presente trabajo se optimizó la transformación biolística de callos embriogénicos escutelares de este genotipo. Primero se identificaron los medios de inducción de callo escutelar (SCIM) más promisorios para la regeneración embriogénica *in vitro* de *Klein Brujo*. Luego, se co-bombardearon embriones de *Klein Brujo* y de *Bobwhite*, una línea altamente transformable, en medios seleccionados con partículas de oro (1 µm) recubiertas de dos plásmidos. Un plásmido contuvo dos genes, el marcador *gfp* (vinculado a *CaMV35S*) y el de selección *bar* (resistencia a fosfinotricina: PPT, vinculado a *Ubi1*) y el segundo al gen *ipt* (isopenteniltransferasa), controlado por el promotor *HvS40* o *SAG12*. Eficacias de transformación de hasta 16% con *Klein Brujo* y 6% con *Bobwhite,* se obtuvieron con embriones pre-cultivados en SCIM por 96 h y sujetos a tratamiento osmótico pre- (4-5 h) y post-bombardeo (16 h). Los callos transgénicos y las plantas regeneradas *in vitro* se identificaron por la detección de la expresión de GFP y la resistencia a PPT. Se transplantaron a suelo, 103 líneas transgénicas de *Klein Brujo* –mucho más que de *Bobwhite* (12 líneas- con frecuencia en un lapso de sólo nueve semanas. La frecuencia de co-transformación de *gfp* y/o *bar* e *ipt* superó el 97% para ambos genotipos, y los tres genes co-segregaron en individuos seleccionados. El gen *ipt* fue estructuralmente estable hasta en la generación T_3, mientras que *gfp* y *bar* fueron susceptibles al silenciamiento.

Palabras clave: Parámetros de bombardeo de partículas; co-transformación; proteína verde fluorescente; resistencia a fosfinotricina; segregación del transgén.

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INTRODUCTION

Wheat is considered to be the world's most important crop, but it has lagged behind the other major cereal crops in terms of the development of efficient transformation methods (Harwood, 2011). The widely employed method of *Agrobacterium*-mediated gene transfer is useful only with particular cultivars of wheat, whereas biolistic transformation is suitable for a wide range of wheat genotypes (Sparks and Jones, 2009). A more robust technology for the generation of transgenic wheat is required for routine application to commercial cultivars that exhibit different genetic backgrounds of locally adapted genotypes (Bhalla, 2006). Breeding programs, for instance, require large numbers of transformed lines to select those showing stable integration and a high level of expression of the transgene. This requirement can be met either by increasing the efficiency of the gene transfer itself or by shortening the time required for the regeneration of transgenic plants (Altpeter *et al.*, 1996). The efficiency with which wheat can be transformed biolistically depends on the genotype (Takumi and Shimada, 1996; Iser *et al.*, 1999; Lazzeri and Jones, 2009) and on a number of other factors that are still poorly understood (Klein and Jones, 1999). These factors can be categorized into biological and physical parameters. The biological parameters are those associated with the target tissue, which is generally the scutellum of immature embryos, and with the medium employed in the *in vitro* culture of the bombarded embryos (Sahrawat *et al.*, 2003). The physical, or ballistic, parameters comprise the type, size and density of the bombardment particle, together with the propellant force and distance to the target that determine the impact intensity of the particle. Efficient transformation involves balancing the point at which maximum possible DNA introduction with minimum cell death resulting from the bombardment (Klein and Jones, 1999). The various biological and physical factors interact, making their empirical optimization difficult. Particular studies have generally focused on optimizing only one parameter, *e.g.*, acceleration pressure (Becker *et al.*, 1994), pre-culture prior to bombardment (Takumi and Shimada, 1996), type and concentration of auxin in the culture medium (Barro *et al.*, 1998) and exposure to pre- or post-bombardment osmotic treatment (Gil-Humanes *et al.*, 2011). Less frequently, combinations of two parameters, *e.g.*, distance to the target tissue and acceleration pressure (Iser *et al.*, 1999), particle size and distance to the target tissue (Jordan, 2000) and particle size and acceleration pressure (Pellegrineschi *et al.*, 2000) have been analyzed. Variations of different parameters of a standard transformation procedure have also been assessed independently with regard to transient or stable expression and the optimized individual parameter values combined in new transformation protocols (Rasco-Gaunt *et al.*, 1999; 2001; Yao *et al.*, 2007).

The methodology employed for selecting transgenic plants is a key factor in wheat transformation (Fadeev *et al.*, 2006). Efficient, cost-effective transformation systems must both accurately select transgenic plants and prevent non-transgenic plants from "escaping" detection, as identification of the latter entails time-consuming additional analysis (Harwood, 2011). The genes *uidA* (encoding β-glucuronidase) and *bar* (encoding phosphinothricin acetyl transferase, which confers resistance to phosphinothricin: PPT, the active ingredient of the herbicide BASTA) are frequently introduced together into target tissue for selection purposes. A major drawback in the use of this combination is, however, the incidence of numerous escape plants, coupled with the lethal nature of the assay for the product of the scorable *uidA*-gene (Sahrawat *et al.*, 2003). The non-destructive reporter GFP (green fluorescent protein, encoded by the *gfp*-gene) has also been successfully used in wheat transformation (Jordan, 2000; Huber *et al.*, 2002; Fadeev *et al.*, 2006). Transgenic wheat plants have been selected on the basis of GFP expression alone, although the inclusion of an antibiotic resistance gene improved the efficiency of the selection (Jordan, 2000). According to Jordan (2000), transformation with *gfp* and a selection gene enables GFP-expressing plants to be recovered from large masses of shoots and minimizes the likelihood of non-transgenic escape plants avoiding detection. The selection agent generally employed is PPT, which is applied to the transformation target either immediately after bombardment or during callus formation (Altpeter *et al.*, 1996; Becker *et al.*, 1994; Huber *et al.*, 2002) or culture in regeneration medium (Jordan, 2000; Rasco-Gaunt *et al.*, 2001; Pellegrineschi *et al.*, 2002; Gil-Humanes *et al.*, 2011). Efforts have also focused on reducing the time required for the establishment of self-sufficient transgenic plants on soil (Altpeter *et al.*, 1996).

The aim of the present investigation was to optimize the biolistic transformation of immature embryos of

the Argentine spring wheat genotype *Klein Brujo* and the selection and growth of transgenic plants from the bombarded embryos. We had previously found *Klein Brujo* to be the most promising of 22 Argentine spring wheat varieties for transformation studies based on embryogenic scutellar callus production and subsequent plant regeneration (Souza Canada and Beck, 2013). We first identified scutellar callus induction media best suited to the formation of embryogenic callus from immature *Klein Brujo* embryos and the regeneration of plants from it. These media were then used to ascertain the combinations of biological and physical parameter characteristics most amenable to the biolistic co-transformation of the immature embryos with the *gfp*- and *bar*-genes, as well as with *ipt*, which encodes isopentenyltransferase, as a candidate gene (manuscript in preparation). Transformation efficiency and transgenic plant production with *Klein Brujo* were compared with those of the wheat genotype *Bobwhite* SH 9826, which had been found to respond particularly well to biolistic transformation (Pellegrineschi *et al.*, 2002). These investigations produced 103 independent primary transgenic lines of *Klein Brujo* and 12 of *Bobwhite*, which we characterized for the transgene presence and inheritance.

MATERIALS AND METHODS

Plant material

Seeds of the spring wheat cultivars *Klein Brujo* (Souza Canada and Beck, 2013) and *Bobwhite* SH 98 26 (Pellegrineschi *et al.*, 2002) were obtained from Criadero Klein S.A. (Alberti, Buenos Aires province, Argentina) and the International Maize and Wheat Improvement Center (CIMMYT: El Batan, Mexico), respectively. Donor plants were grown from these seeds throughout the year in the greenhouse as described by Souza Canada and Beck (2013).

In vitro culture

Immature zygotic embryos from suitably developed seeds were excised as described by Souza Canada and Beck (2013) and placed on solidified sterilized callus induction medium. The embryos were approximately 1.5 mm in length, with an approximately 1.3 mm long, transparent scutellum; they were at the developmental stage H specified by Souza Canada and Beck (2013), which corresponds to the developmental stage II described by He *et al.* (1986) and the early to middle milking stages 73-76 of the Zadoks´ scale (Zadoks *et al.*, 1974).

Two basic types of scutellar callus induction medium (SCIM) were employed to induce and maintain callus growth of the excised immature embryos. One was ML3 basal medium (Viertel and Hess, 1996), containing 3 % maltose (w/v) and solidified with 0.2 % Gelrite. The second was MSD basal medium (Barcelo and Lazzeri, 1995) with the following modifications: 118 µM $MnSO_4 \cdot H_2O$, 30 µM $ZnSO_4 \cdot 7\ H_2O$ and 102 µM H_3BO_3, in addition to the double original content of amino acids and maltose instead of sucrose. The maltose content of this medium (termed "MB" in the following), which was solidified with 0.8 % (w/v) agar, was either 3 % (medium "MB3") or 9 % (w/v) (medium "MB9"). The supplementation of ML3, MB3 and MB9 with 1 or 2 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D) resulted in six SCIMs for testing (ML3-1 and -2, MB3-1 and -2, MB9-1 and -2).

Five replicates of 25 excised embryos were incubated on solidified callus induction medium in Petri dishes for each individual experiment. After three weeks in darkness at 25° C, callus induction and cell proliferation on the scutellum surface and precocious germination were assessed for each embryo under a stereomicroscope. Embryos with compact, nodular embryogenic calli were transferred to Petri dishes with modified MSB medium and cultivated on this for three weeks for plant regeneration, whereupon the regenerative capacity of the callus was evaluated and the number of plantlets regenerating from it was determined. Regenerating plantlets were transferred directly to autoclaved substrate (70 % compost and 30 % clay) when they possessed a well-developed root system. If the root system was underdeveloped, the plantlets were first cultivated for 2-3 weeks on rooting medium 190-2 (Zhuang *et al.*, 1984). After acclimation to the substrate in a culture room, the plantlets were transferred to a greenhouse for *ex vitro* culture. These procedures are described in detail in Souza Canada and Beck (2013).

DNA plasmid vectors and particle bombardment

The plasmid employed in all experiments was the dual expression vector pGFPBAR (Huber *et al.*, 2002). This contains the *bar*-gene (Thompson *et al.*, 1987) under the control of the maize ubiquitin-promoter and intron-1-complex (*Ubi1*) (Christensen *et al.*, 1992), as well as a chimeric S65T-p*gfp*-intron gene (Pang *et al.*, 1996)

driven by the cauliflower mosaic virus *CaMV35S*-promoter (Odell *et al.*, 1985). It was delivered in co-transformation experiments at an equimolar ratio of 1:1 with one of two different plasmids containing the *ipt*-gene. One of these plasmids was pSG516 (Gan and Amasino, 1995), in which the *ipt*-gene (Li *et al.*, 1992) was linked to the *SAG12*-promoter. The second, pS40-IPT, was constructed from pSG516 and pS40-GUS (kindly donated by Prof. Dr. K. Krupinska, Christian-Albrechts-Universität, Kiel, Germany, containing the *uidA*-gene together with the *HvS40*-promoter) by digesting both with *PaeI* and *NcoI* to remove the inducible *SAG12* and *HvS40* promoters. The *HvS40*-promoter was then coupled to the *ipt*-gene of pSG516 to form the new plasmid pS40-IPT (Souza Canada, 2012). Schematic representations of the three plasmid vectors are shown in Figure 1.

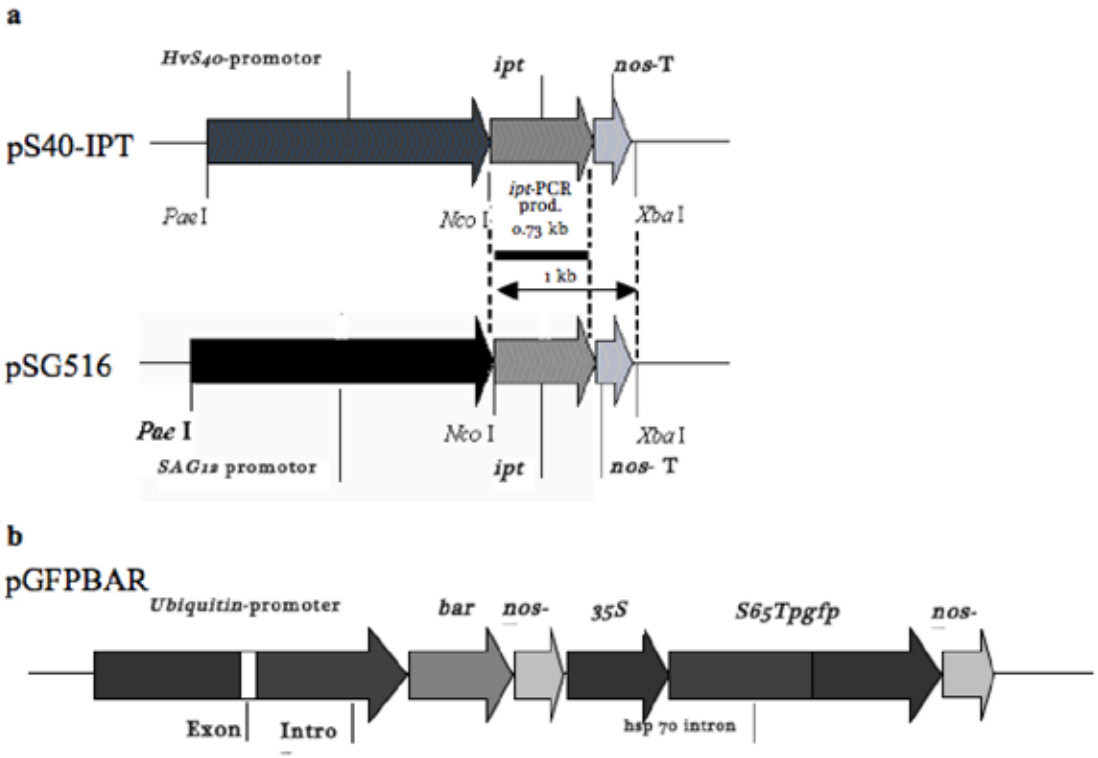

Figure 1. Plasmids used for wheat transformation. a: pS40-IPT (5.7 kb) and pSG516 (5.9 kb) each containing the *ipt* gene (0.7 kb) under the control of the *HvS40* (2 kb) and *SAG12* (2.1 kb) promoters, respectively, and flanked downstream by the 0.3 kb nopaline synthase 3´-terminator sequence (*nos*-T). The PCR-product obtained with *ipt*-primers is designated by a bold bar, and the fragment released by *NcoI/XbaI* digestion is indicated by a double arrow. b: pGFPBAR (8.8 kb). The *CaMV* 35S (35S) promoter governs the *gfp* complex containing the maize heat-shock protein 70 (*hsp* 70) intron and the synthetic *gfp*-gene containing the potato ST-LS1-intron (*S65Tpgfp*). The *bar*-gene was under control of the maize *ubiquitin* promoter followed by exon-1 and intron-1 of the maize *ubiquitin* gene.

Plasmid DNA (5 µg in each case) was precipitated onto a 3 mg suspension of gold particles and the DNA-gold suspension (6 µl) was loaded onto the center of the particle gun macrocarrier as described by Iser *et al.* (1999), whereby the precipitation mixture was finger-vortexed for 6 min instead of mechanically vortexed for 3 min, and then incubated at room temperature for 20 min followed by a brief centrifugation. For each transformation experiment 4-5 replicates of 30-35 immature wheat embryos were arranged in the centers of 90 mm Petri dishes with SCIM within rings of 2.4 cm diameter. They were bombarded with the DNA-coated gold particles using the PDS 1000/He particle gun (BioRad, Germany).

Embryo bombardments were carried out at various settings of a number of variable culture-relevant (termed "biological") and ballistic, or "physical" parameters (Table 1). The basic biological parameter was the particular SCIM on which the embryos were cultured. The excised embryos were pre-cultured on this medium for up to 120 h prior to bombardment and, were in most cases also incubated on their respective SCIM supplemented with 0.5 M mannitol for several hours prior to and subsequent to the bombardment. They were then transferred to fresh SCIM without mannitol. The physical parameters varied with regard to the size of the gold particles, the distance from the microcarrier launch assembly to the target tissue and the acceleration pressure. The actual parameter variant combinations employed in the transformation experiments are specified in the respective sections of the Results.

Table 1. Biological and physical parameters examined in the biolistic transformation of excised wheat embryos; SCIM: Scutellar callus induction medium

Parameter	Variants employed
Biological	
Scutellar callus induction medium (SCIM)	ML3-1, MB3-1, MB9-1
Osmotic treatment- 4-5 h pre-bombardment/16 h post-bombardment	SCIM with or without 0.5 M mannitol
Pre-culture on SCIM prior to bombardment (h)	0, 20, 72, 96, 120
Physical	
Gold particle diameter (µm)	0.6, 1.0
Target distance (cm)	6, 9, 12
Acceleration pressure (psi)	900, 1,350

Selection of transgenic plants

The transformation protocol used is outlined in Figure 2. GFP expression was monitored from 48 h after the bombardment onwards using a MZFLIII fluorescence stereomicroscope (Leica, Germany) with an excitation filter BP 490-550 nm (Leica) and documented with an AxioXam CCD-camera and corresponding computer program (Zeiss, Germany). Scutellar calli growing on SCIM were scored for GFP-expression 10-12 days after bombardment. All viable calli present at the end of the three-week embryogenic scutellar callus formation phase, and any shoots having already developed from them, were divided into three groups. Group A was made up of calli and shoots exhibiting stable GFP expression. Group B included calli and shoots that had initially exhibited GFP expression, but had lost it by the end of the callus formation phase. Group C was composed of calli and shoots that had never exhibited any GFP fluorescence. Calli and shoots of groups A and B were cultivated for shoot regeneration on modified MSB medium (Souza Canada and Beck, 2013) in Petri dishes for three weeks without PPT (round R in Figure 2). Those of group C were cultivated under the same conditions, but with 4 mg/ml PPT in the medium (round P in Figure 2). Calli and shoots or plantlets (shoots in the process of developing roots) were then transferred to fresh shoot regeneration medium in test tubes and cultured for another three weeks. No PPT was included in the medium for shoots and plantlets from group A that still exhibited GFP fluorescence (round S in Figure 2). The medium for the remaining shoots and plantlets from group A, which no longer exhibited GFP fluorescence, as well as for all of the GFP-negative shoots and plantlets from groups B and C was supplemented with 4 mg/l of PPT (rounds T in Figure 2). Plantlets possessing a well-developed root system at the end of the shoot regeneration phase were transferred directly to autoclaved substrate (see above) and transferred to

a greenhouse. Shoots not possessing well-developed roots were first cultivated for 2-3 weeks on rooting medium 190-2 (Zhuang *et al.*, 1984) before being transferred to the substrate. In these cases the medium for GFP-expressing group A plantlets was free from PPT (round V in Figure 2), whereas it contained 4 mg PPT/l for all GFP-negative plantlets (rounds E in Figure 2).

After a few weeks of *ex vitro* culture, a small disk of filter paper imbibed with a 3 mM PPT solution was attached to two or three leaves of the putatively transgenic plants and to non-transformed control plants with a paper clip. This treatment was performed three times with an interval of one week between the treatments. This test confirmed transgenic PPT resistance in the plants when no discoloration of the treated leaf area occurred. The "transformation efficiency" of a particular experiment was defined as the number of identified transgenic plants expressed as percent of the total number of bombarded embryos.

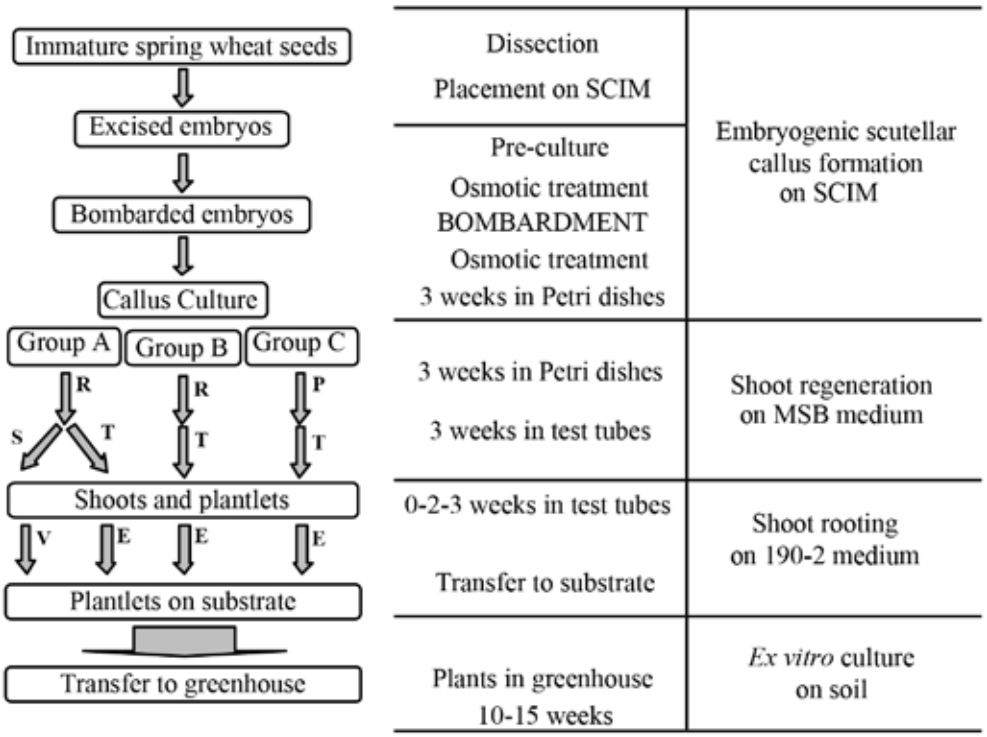

Figure 2. Production of transgenic *Klein Brujo* and *Bobwhite* spring wheat plants. Group A refers to calli and shoots expressing GFP at the end of the scutellar callus formation phase, whereas those of group B no longer showed initially present *gfp* expression and those of group C had never shown any evidence of *gfp* expression. The selection rounds designated by R, S and V were carried out in the absence of PPT, whereas in those indicated by P, T and E the medium contained 4 mg/l PPT. *In vitro* culture was from 63 up to 84 days, depending on the length of the shoot rooting phase.

Transgene analysis

Genomic DNA was extracted from young leaves according to Souza Canada (2007). PCR was carried out at least three times with each extract to determine the presence of the *ipt*-gene in plants exhibiting GFP expression and/or PPT resistance. Controls were performed with 0.2 ng of the plasmids containing this gene. PCR was run in a reaction volume of 30 µl containing 10-50 ng wheat genomic DNA, 3 µl 10x Long PCR buffer (Sambrook and Russell, 2001), 2.5 mM $MgCl_2$, 160 µM of each of the 4 dNTPs, 0.5 µM of the two *ipt*-specific primers and 1 U Taq DNA polymerase (Fermentas, Germany). The *ipt*-gene was amplified by using 5'-ACC CAT GGA CCT GCA TCT A-3' as the forward primer and 5'-GGA GCT CAG GGC TGG CGT AAC C-3' as the reverse primer at an annealing temperature 63° C. DNA was denatured at 95° C for 4 min, followed by amplification cycles of 1 min at 95° C, annealing for 1 min and heating at 72° C for 1 min. After 30 cycles the PCR product (730 bp) was separated by electrophoresis in 0.8 % w/v agarose gels, stained with ethidium bromide and visualized under UV light. Transgene integration was confirmed by *Southern* blot analysis. Genomic DNA (25-30 µg) was digested overnight at 37° C with 0.32 U/µg of each of the restriction enzymes *NcoI* and *XbaI*. For positive controls 10 ng of the *ipt*-bearing plasmids were digested; negative controls were performed with DNA of non-transformed plants. Digested genomic DNA was fractionated by electrophoresis in 0.8 % (w/v) agarose gels, blotted onto a positively charged nylon membrane (B/Plus, Biodyne, Germany) and hybridized with a biotin-11-dUTP-labelled probe at 52° C overnight in hybridization buffer. Non-radioactive labeling of the PCR products was performed with a Fermentas Biotin DecaLabel DNA Labeling Kit according to the manufacturer's instructions. Labelling was detected with streptavidin-alkaline phosphatase conjugate (Streptavidin AP, Novagen, Germany), using the DIG Luminescence Detection Kit from Roche (Germany). Phosphatase activity was visualized by exposure to X-OMAT AR film (Kodak, Germany) for 20 min up to five days, depending on the signal strength.

Transgene inheritance

The inheritance of the transgenes was analyzed by testing for the presence of *ipt* by PCR and for the expression of *gfp* and *bar* by monitoring GFP fluorescence and resistance to PPT in T_1, T_2 and T_3 progeny. Segregation ratios were calculated from these data. T_1 progenies were obtained by selfing T_0 transgenics, excising immature embryos from them at the growth stage 75 according to Zadoks *et al.* (1974), culturing the embryos for five days on WH medium (Wagner and Hess, 1973) supplemented with 3 % (w/v) sucrose and 0.1 mg/l indole acetic acid and solidified with 0.8 % agar and subsequently transferring them to substrate for *ex vitro* culture (see above). The T_2 and T_3 progenies were obtained in an analogous manner.

Statistical analysis

Data were evaluated by the X^2 test of independence (contingency table analysis), variance analysis and calculation of standard deviation. If necessary, the *post hoc* test was carried out between the samples from the six culture conditions, as follows: among the same medium containing different concentrations of 2,4-D and among different media containing the same concentration of 2,4-D. Subsequently, the significance calculated was compared with the manually corrected value (Bonferroni-Correction according to Holm). Segregation of transgene expression in sexual progenies was analyzed by the χ^2 test for statistical deviation from the Mendelian ratio for single locus integration. The statistical software package SPSS 13.0 (SPSS 2004) was used for all analyses.

RESULTS

In vitro culture of immature Klein Brujo embryos

The scutella of embryos excised from immature seeds of the spring wheat cultivar *Klein Brujo* produced both friable, watery and translucent non-embryogenic callus and compact, nodular embryogenic callus on each of the six SCIMs. The embryogenic callus formed on ML3 medium was white to pale yellow (Figure 3a), whereas it was bright yellow on MB3 medium (Figure 3b) and darker yellow on MB9 medium (Figure 3c). The *in vitro* culture performance of the embryos on the six media is documented in Table 2.

The percentages of embryos exhibiting embryogenic scutellar callus formation differed significantly after three weeks of incubation on the six SCIMs (Chi-square-test X^2= 151.2; df= 5; $p<0.001$). They ranged from 42.2 % on the medium MB3-2 to 98.6 % on MB3-1 (Table 2). The highest rates of callus formation were found on the ML3-, MB3- and MB9- media containing 1 mg/l 2,4-D (ML3-1, MB3-1 and MB9-1). The rate on ML3-2 with 2 mg/l 2,4-

D was not significantly different to that on ML3-1, but the rates on MB3-2 and MB9-2 with 2 mg/l of the auxin were significantly lower than those on the corresponding MB-media with the lower hormone concentration (MB3-1 and MB9-1).The extent to which embryogenic callus proliferated on the scutellum also differed significantly with the type of SCIM (Chi-square-test X^2= 54.4; df= 5; p<0.001). The percentage of the scutellum surface covered by embryogenic callus after three weeks of culture ranged from 59.5 % on the medium MB9-1 to 95.7 % on MB3-1 (Table 2).

Immature embryos germinated precociously to significantly different extents during the course of three weeks on all of the six callus induction media (Chi-square-test X^2= 26.9; df= 5; p<0.001). The frequency of precocious germination ranged from 0.61 % on ML3-1 to 10.9 % on MB9-2 (Table 2). However, the respective differences were not always significant and could not be related to different 2,4-D or maltose concentrations.

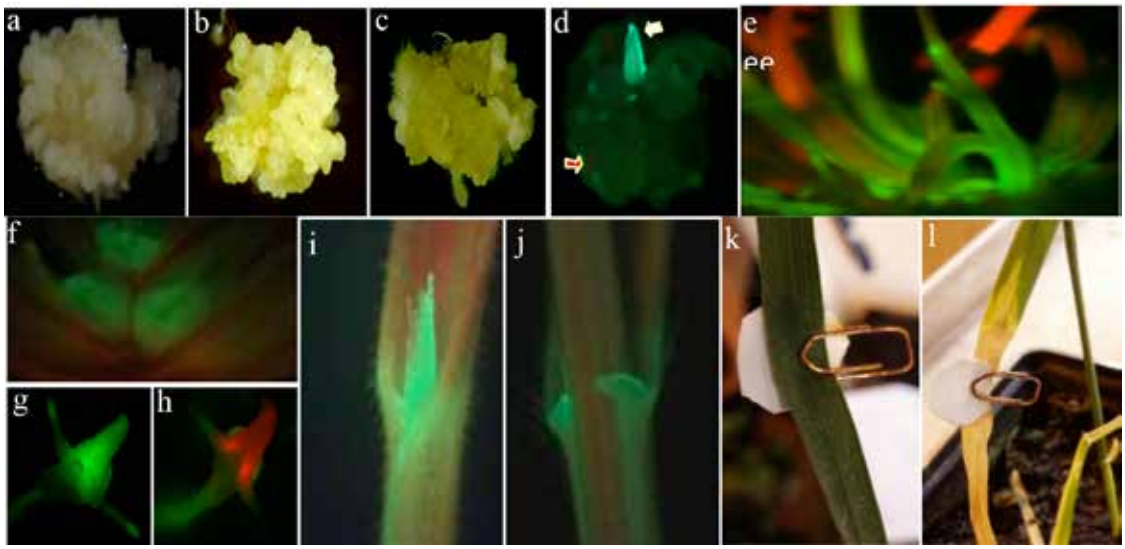

Figure 3. Callus formed on excised immature cv. *Klein Brujo* wheat embryos and transgenic structures and plants developed from callus produced by embryos bombarded with pGFPBAR and pS40-IPT. a, b and c: Embryogenic calli covering the scutellum surface after three weeks of incubation of the embryos on ML3-1, MB3-1 and MB9-1 medium, respectively. d-j: Expression of the *gfp*-gene in tissues and shoots; d: shoots showing and not showing GFP-expression as indicated by white and red arrows, respectively, three weeks after bombardment, e: plantlets developing on *in vitro* regeneration medium, f: transgenic grains, g and h: immature embryos of transgene (g) and azygous (h) T1 progenies, i: transition from leaf sheaths to the blade wrapping a young leaf, j: auricles and stem. k and l: Sections of mature leaves of T1 wheat plants one week after the last of three applications of 3 mM PPT on a filter paper disc attached to the leaf by a paper clip; k: no leaf discoloration on a homozygous leaf (resistant to PPT), l: leaf yellowing progressing from the paper disk on an azygous leaf (lacking resistance to PPT).

Table 2. *In vitro* culture performance of excised immature embryos of *Klein Brujo* on six scutellar callus induction media

	SCIM					
	ML3-1	ML3-2	MB3-1	MB3-2	MB9-1	MB9-2
Scutellar callus formation (% of excised embryos) [3]	84.8 ± 12.1^{af}	72.5 ± 12.6^{aj}	98.6 ± 4.3^{bgh}	42.2 ± 51.3^{ckl}	87.7 ± 6.7^{dfi}	54.3 ± 16.5^{ejl}
Callus proliferation on the scutellum surface (% cover)[4]	77.0 ± 18.1^{ae}	71.2 ± 6.4^{ai}	95.7 ± 44.3^{bfg}	61.6 ± 14.9^{cij}	59.5 ± 7.1^{deh}	64.5 ± 10.6^{dij}
Precocious germination (% of embryos)[5]	0.61 ± 2.2^{ad}	2.5 ± 3.3^{ae}	5.5 ± 17.1^{bd}	2.0 ± 5.4^{beg}	2.5 ± 4.4^{cd}	10.9 ± 8.9^{cfg}
Plant regeneration capacity (%)[1, 6]	100^{af}	76.3 ± 22.7^{bj}	100^{cfh}	72.1 ± 7.1^{djl}	55.7 ± 24.5^{egi}	51.3 ± 2.1^{ekl}
Number of regenerated plants per embryo [7]	9.5 ± 6.4^{ae}	5.6 ± 3.5^{bh}	4.5 ± 3.4^{cfg}	3.9 ± 2.5^{chj}	4.4 ± 2.5^{dfg}	2.9 ± 2.1^{dij}
Culture efficiency (%)[2,8]	84.8 ± 6.0^{ag}	55.3 ± 17.5^{bk}	98.6 ± 2.1^{chi}	30.4 ± 29.1^{dlm}	48.8 ± 15.6^{ehj}	27.8 ± 9.3^{flm}

[1] Percentage of callus-forming embryos producing regenerants
[2] Percentage of cultured embryos producing regenerative calli (nodular callus exhibiting green spots)
[3, 4, 5, 6, 7, 8] Post hoc tests (pairwise comparison): Means followed by the same letter are not significantly different at the 5% level

Plantlet regeneration

Embryogenic calli from immature embryo scutella were transferred to shoot regeneration medium. Their ability to regenerate shoots and plantlets (shoots with developing roots) during a further 3 weeks of culture (the "plant regeneration capacity" in Table 2) differed significantly with the medium having been used to induce callus formation (Chi-square-test X^2= 98.1; df= 5; $p<0.001$). The frequency of plant regeneration ranged from 51.3 % for calli formed on MB9-2 to the maximum possible value of 100 % for calli formed on each of ML3-1 and MB3-1, which both contained 3 % maltose and 1 mg/l 2,4-D. The regenerative capacities of calli on ML3- and MB3-media with 2 mg/l 2,4-D (ML3-2 and MB3-2) were significantly lower than with 1 mg/l of the auxin, and those on the two MB- media containing 9 % maltose (MB9-1 and MB9-2) were the lowest observed, irrespective of the 2,4-D concentration.

The number of plantlets that regenerated from each cultured embryo after 3 weeks on shoot regeneration medium also differed appreciably according to the callus induction medium on which the calli had originally formed (Kruskal-Wallis-test X^2= 48.4; df= 5; $p<0.001$), ranging from 2.9 on MB9-2 to 9.5 on ML3-1 (Table 2). The highest numbers (9.5 and 5.6 plantlets) corresponded to calli formed on the two ML-media, and these numbers were significantly higher than for the MB-media. The 2,4-D concentration of 1 rather than 2 mg/l in the SCIM led to more plantlets regenerated per embryo only for calli formed on ML3 medium. The number of plantlets regenerated upon callus formation on MB-medium was not significantly influenced by the maltose supply to the medium.

Many of the young plantlets developing from calli possessed well-developed roots following culture on shoot regeneration and rooting media. Random individuals were transferred to soil and cultivated first for 1-2 weeks under *in vitro* culture ambient conditions and thereafter *ex vitro* in a greenhouse. These plants showed normal morphological development and maturation and set viable seeds after approximately three months.

Culture efficiency

The percentage of immature embryos developing regenerative calli (calli from which plantlets regenerated) designates the efficiency of the culture in propagative

potential from the embryos. As shown under "culture efficiency" in Table 2, this percentage differed significantly among the six callus induction media (Chi-square-test X^2= 162.4; df= 5; p<0.001). Culture efficiency attained almost the maximum possible value on MB3-1 (98.4 %), and that on ML3-1 (also well over 80 %) was also much higher than on any of the other media. MB medium gave a better result than ML medium at a 2,4-D concentration of 1 mg/l (compare MB3-1 and ML3-1), but ML was better at 2 mg/l of the auxin (compare MB3-2 and ML3-2). Culture efficiency was always significantly better with1 mg/l than at 2 mg/l 2,4-D. A maltose concentration of 3 % in MB medium resulted in significantly better culture efficiency than did 9 % and1 mg/l 2,4-D (compare MB3-1 and MB9-1), but not with 2 mg/l of the auxin (compare MB3-2 and MB9-2).

Choice of medium for biolistic experiments

Overall, media MB3-1 and ML3-1 best induced the formation, and supported the proliferation, of embryogenic scutellar callus with excellent plant regeneration capacity and a high culture efficiency. These two media were accordingly chosen as SCIMs for the biolistic experiments. MB9-1 was also chosen for these experiments despite its considerably poorer culture performance data on account of its greater osmotic strength (due to its maltose concentration of 9 % at the same 2,4-D concentration of 1 mg/l), which had been shown to benefit callus formation on and plant regeneration from bombarded embryos (Rasco-Gaunt *et al.*, 2001).

Transformation by particle bombardment

Immature embryos of the genotype *Klein Brujo* were co-bombarded with the plasmids pGFPBAR and pS40-IPT under combinations of the biological and physical parameter variants listed in Table 1. The embryos were maintained on the SCIM on which they had been bombarded, and scutellar calli, shoots and plantlets formed from them were subsequently cultured on shoot and root regeneration medium and substrate as outlined in Figure 2. Calli and plants regenerated from them were assessed for successful transformation by testing for GFP fluorescence and resistance to PPT. Transformants were obtained only when the embryos were pre-cultured on SCIM for at least 20 h and subjected to pre- and post-bombardment osmotic treatment with 0.5 M mannitol. Using ML3-1 medium transgenic plants were obtained only under a single combination of bombardment parameter settings; pre-culture for 72 h bombardment with 1.0 μm gold particles, acceleration pressure of 1350 psi and a target distance of 9 cm resulted in a culture efficiency of 19.2 % and an overall transformation efficiency of 1.9 % (best value for an individual bombardment 4.5 %). In contrast, a variety of combinations of bombardment parameters led to the production of transgenic plants for embryos cultivated on MB3-1 or MB9-1 medium. Culture efficiencies ranged between 10.8 % and 69.8 % on MB3-1 and between 29.3 % and 67.7 % on MB9-1. Transformation efficiencies ranged up to 10 % (best value for an individual bombardment: 16.4 %) for embryos cultivated on MB3-1 and up to 4.8 % (best individual value: 11.9 %) for those on MB9-1. The highest culture and transformation efficiencies were obtained with the same combination of physical bombardment parameters on both MB3-1 and MB9-1. In general, increasing periods of pre-incubation led to progressively higher culture and transformation efficiencies, with the best results being obtained after 96 or 120 h of pre-culture. Transgenic plants were obtained for 11 of the 12 physical bombardment parameter combinations when MB3-1 was used as the SCIM (96 h of pre-culture; Table 3), but only for five of the 12 combinations tested with MB9-1 (not shown). Both the highest overall and the best individual bombardment efficiencies using MB3-1 as the SCIM were obtained with 1.0 μm gold particles delivered at an acceleration pressure of 1350 psi and a target distance of 9 cm (Table 3). The best efficiencies on MB9-1 were also observed for the 1.0 μm gold particle size and 1350 psi acceleration pressure, but at a target distance of 6 cm following 120 h of pre-culture. Relatively high transformation efficiencies ranging from 4.2 % to 8.6 % were also noted for culture on MB3-1 with the smaller (0.6 μm) gold particles at acceleration pressures of both 900 and 1350 psi and target distances of both 6 and 9 cm (Table 3).

On the basis of these results, excised immature *Bobwhite* embryos were also pre-cultured for 96 h on each of MB3-1 and MB9-1, co-bombarded with pGFPBAR and pSG516 under osmotic treatment with 0.5 M mannitol and combinations of the gold particle sizes, acceleration pressures and target distances listed in Table 1, and cultured further as for *Klein Brujo*. Better culture efficiencies were obtained with the bombarded *Bobwhite* embryos on MB3-1 (1.2-45 %: Table 3) than on MB9-1 (0-32.8 %). Transgenic plants were obtained with four of the

12 combinations of physical bombardment parameters tested with MB3-1 as the SCIM (Table 3), but with only three of the 11 combinations tested with MB9-1. The transformation efficiencies for the successful physical bombardment parameter combinations reached 4.1 % for culture on MB3-1 (Table 3) and 1.6 % respective of MB9-1. As shown in Table 3, both the culture and efficiencies attained with *Bobwhite* were lower than those determined for *Klein Brujo*.

Selection of transgenic plant material

Scutellar calli developing on the bombarded embryos (some already bearing rudimentary shoots; Figure 3d) were assigned to groups A, B or C according to GFP expression at the end of the 3-week scutellar callus formation phase (Figure 2). Further monitoring of GFP fluorescence (in group A) or resistance to PPT led to the isolation of 103 independent primary transgenic lines of *Klein Brujo* and 12 of *Bobwhite*. Eighty-one of the *Klein Brujo* and eight of the *Bobwhite* lines originated from callus culture on MB3-1. The remaining 22 *Klein Brujo* lines stemmed from bombarded embryos cultured on ML3-1 and MB9-1, and the remaining four *Bobwhite* transgenics derived from callus culture on MB9-1. Thirty-two of the 103 transgenic *Klein Brujo* plants derived from group A, 35 from group B and 36 from group C. Five, four and three of the 12 transgenic *Bobwhite* plants stemmed from the groups A, B and C, respectively. Interestingly, 22 of the 103 transgenic *Klein Brujo* plants were first detected as GFP-expressing plantlets emerging from group B and C cultures at the end of either the shoot regeneration or the shoot rooting phase (Figure 2 and 3e), as they had previously been obscured by other tissues. Most of the transgene plants growing *ex vitro* on substrate were phenotypically normal, but the flowers of some spike of some lines were infertile and accordingly showed a reduced grain production.

All but three of the 103 GFP- or PPT- positive *Klein Brujo* plants (Table 3) and all of the 12 transgenic *Bobwhite* plants showed the presence of the *ipt*-gene upon PCR-analysis (Figure 4). This corresponded to a frequency of co-transformation of the *gfp*- and/or *bar*-genes (from pGFPBAR) and the *ipt*-gene (from pS40-IPT or pGS516) of 97.1 % for *Klein Brujo* and 100 % for *Bobwhite*.

In some instances GFP fluorescence observed at 10-12 days after bombardment was lost during the course of scutellar callus formation and plant regeneration. Twenty-seven of the 103 transgenic *Klein Brujo* plants exhibited

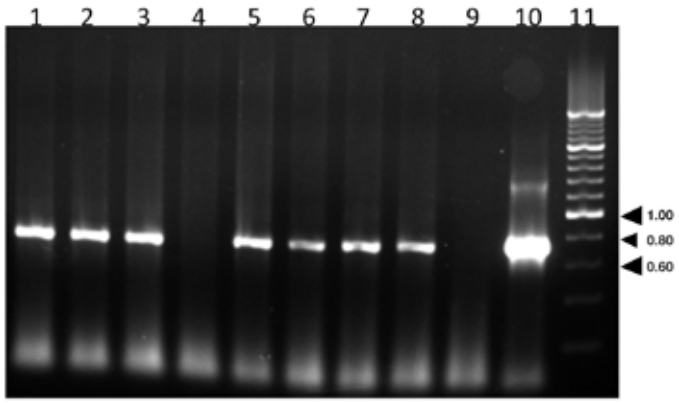

Figure 4. PCR analysis of genomic DNA extracted from control and independent wheat T_0-lines. Amplification of *ipt*-gene was carried out with *ipt*-primers. The *ipt*-PCR product was 730 bp long as shown in Fig 1. Lanes 1-3: cv. *Bobwhite* transformed with pSG516, lane 4: non-transformed cv. *Bobwhite*, lanes 5-8: cv. *Klein Brujo* transformed with pS40-IPT, lane 9: non-transformed cv. *Klein Brujo*, lane 10: plasmid pS40-IPT (positive control), lane 11: O´RangeRuler 200 bp DNA Ladder (Fermentas).

this phenomenon, of which 25 belonged to group B and had thus lost the ability to express GFP during scutellar callus formation. The four group B transgenic *Bobwhite* plants also reflected early loss of GFP expression. The two remaining *Klein Brujo* transgenics were group A plants (Table 3) that had lost *gfp*-gene expression by the end of round R of plantlet regeneration (see Figure 2). Since all surviving *gfp*-negative transgenic plants had tolerated treatment with PPT (see Figure 2), they were considered as stably expressing the *bar*-gene.

Selection on the basis of GFP expression alone without the use of any PPT produced 21 transgenic *Klein Brujo* and three transgenic *Bobwhite* plants after only 9 nine instead of the usual 15 weeks of *in vitro* culture. This rapid regeneration was possible because the transgenic shoots had a well-developed root system at the end of the shoot regeneration phase (round S in Figure 2) and thus could be transferred directly to soil without the need for *in vitro* shoot rooting (round V in Figure 2).

Molecular and biochemical analysis

Six independent transgenic T_0 lines of *Klein Brujo* and two of *Bobwhite* were selected for further investigations under the designations KB-1 to KB-6 and Bw-1 and Bw-2, respectively. Southern blot analyses were carried out on these eight lines and on non-transformed plants for

negative controls. When genomic DNA digested with *NcoI* and *XbaI* (Figure 1) was separated electrophoretically and probed with a biotin-labelled *ipt*-coding region fragment (Figure 1), a 1 kb biotin-labelled product was detected for all eight T_0 plants (Figure 5). This confirmed the transgene integration of at least one intact *ipt*-gene into each target tissue genome, corroborating the positive results of the PCR analyses. Reverse transcription PCR provided further confirmation of the presence and expression of the *ipt*-gene in all eight T_0 plants (not shown).

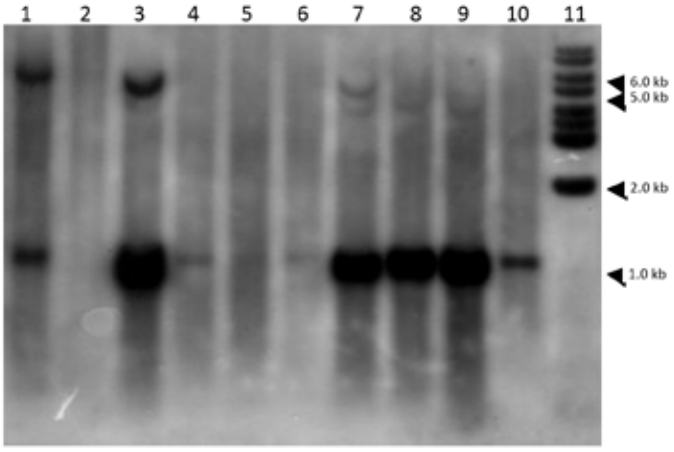

Figure 5. Southern blot hybridization of a biotin-labelled *ipt*-probe to fragmented genomic DNA from six cv. *Klein Brujo* (KB) and two cv. *Bobwhite* (Bw) independent T_o lines transformed by bombardment with the pS4O-IPT and pSG516 plasmids, respectively. Genomic DNA was digested with the restriction enzymes *Nco*I and *Xba*I that released a 1.0 kb plasmid fragment containing the *ipt*-gene and the *nos*-terminator (Fig.1). Lane 1: KB-1, lane 2: negative control (non-transformed plant), lane 3: KB-2, lane 4: KB-3, lane 5: KB-4, lane 6: KB-5, lane 7: Bw-1, lane 8: Bw-2, lane 9: KB-6, lane 10: positive control (plasmid pGS516 containing the 1.0 kb fragment released by digestion with *Nco*I and *Xba*I), lane 11: GeneRuler 1 kb DNA Ladder (Fermentas).

The leaves of the selected six *Klein Brujo* and two *Bobwhite* T_0 plants were also resistant to PPT when a 3 mM PPT solution of the herbicide was applied locally to the plants growing *ex vitro* in the greenhouse. Whereas the leaves of the transgenic plants remained green upon the treatment with PPT, those of non-transformed control plants exhibited considerable damage and browning. The *bar*-gene was always stably expressed up to and during *ex vitro* culture, but not the *gfp*-gene. The KB-3 line stemming from group B transgenics had lost GFP expression during *in vitro* callus formation culture, while the KB-6 line was a group C transgenic that had never discernibly expressed GFP (see Figure 2).

Analysis of transgene inheritance

Each of the eight selected T_0 transgenic lines KB-1 to KB-6 and Bw-1 and Bw-2 were self-pollinated and 20 of the resultant T_1 generation seeds were removed from each line (with the exception of KB-3, from which all of the only nine formed seeds were taken). GFP fluorescence was observed in the seeds of the six GFP-positive T_0 lines (Figure 3f). The seeds were planted in soil and the seedlings sprouting from them were grown to fully developed plants in the greenhouse. Each of the 149 T_1 progeny plants was screened for the presence of the *ipt*-gene by PCR, as well as for GFP expression (Figure 3i and 3j) and PPT resistance (Figure 3k and 3l). The results of this analysis are shown in Table 4. The segregation of *ipt* in the T_1 progeny of all eight T_0 plants showed that this gene was inherited at a 3:1 ratio consistent with a single transgene locus, providing further evidence of stable transformation. However, the phenotypic segregation of GFP expression agreed with the Mendelian 3:1 ratio in the T_1 progeny of only four of the six GFP-positive T_0 lines (Bw-1, KB-1, KB-2 and KB-4), but not in Bw-2 and KB-5. GFP fluorescence or the lack of it in T_1 progeny embryos is shown in Figure 3g and 3h. And although the *bar*-gene was expressed in the young leaves of all of the T_0 plants as shown by resistance to PPT, Mendelian segregation was not evident respective of this gene in the T_1 progeny of either of the two T_0 lines that were *gfp*-negative (KB-3 and KB-6). In no instance was there any evidence of independent segregation of the three transferred foreign genes *gfp*, *bar* and *ipt*. Both *ipt* presence and *gfp* expression was observed in all of the T_1 progeny of only KB-1 and KB-4. Some of the T_1 progeny from the T_0 lines Bw-1, Bw-2, KB-2 and KB-5 were PCR-positive for the *ipt*-gene but not for GFP expression, even though Bw-1 and KB-2 exhibited the Mendelian segregation ratio for GFP expression. Similarly, some of the T_1 progeny from the GFP-negative KB-3 and KB-6 T_0 lines were *ipt*-positive plants that were not resistant to PPT and thus did not express the *bar*-gene.

To investigate the inheritance patterns exhibited by the transgenic T_1 plants, T_2 progeny were grown from seeds of all the 149 T_1 plants considered in Table 4, as described above. The number of T_2 progeny plant individuals

stemming from a particular parental T_1 plant varied between 10 and 79. All of these were screened for *gfp* -and *bar*-gene expression by testing for GFP fluorescence and PPT resistance, and for the presence of the *ipt*-gene by PCR analysis. The number of homozygous, hemizygous, and azygous T_1 plants obtained in terms of *ipt* presence, GFP expression and PPT resistance are shown in Table 4. The analysis of T_3 progeny grown from seeds of the T_2 plants revealed that the *ipt*-gene inherited from hemizygous T_2 plants segregated with the same 3:1 ratio that was observed in the inheritance from the T_1 plants (data not shown), again consistent with a single transgene locus.

The progressive loss of expression of transgenes observed in the T_1 plants was also evident in the successive T_2 and T_3 generations as shown in Table 5. Whereas the T_3 progeny of the homozygous T_2 plants KB-1-C-3, KB-4-B-1 and KB-4-B-3 exhibited stable expression of both the *gfp* and *bar*, all other T_3 progeny reflected a loss of this expression to varying degrees. This loss of expression affected either the *gfp*-gene alone or both the *gfp* and *bar* genes. However, the loss of expression of the *bar*-gene tended to be less pronounced than that of the *gfp*-gene. This was particularly well illustrated in the extreme case of KB-5-R-2, where none of the T_3 plants showed any of the GFP expression shown by their parent, whereas they all exhibited resistance to PPT.

DISCUSSION

Choice of a scutellar callus induction medium for in vitro culture

Successful biolistic transformation of wheat depends on an efficient *in vitro* culture system that supports the development and proliferation of embryogenic callus on the scutellum, promotes the development of shoots from these calli and supports rooting of the shoots to produce plantlets for growth on soil. Demands on such a system are high, as biolistic bombardment inevitably results in some damage, even to successfully transformed cells. Such damage must be compensated for by a suitable scutellar callus induction medium (SCIM) on which the bombarded embryos are initially cultured.

The interplay of basal medium composition, hormone and sugar concentration determining the success of a particular SCIM in supporting callus formation and plant regeneration from non-bombarded embryos was not necessarily effective in facilitating the production of transgenic plants from bombarded *Klein Brujo* embryos. The fact that transgenic plants were obtained only when the excised embryos were pre-incubated on the SCIM prior to the bombardment and also subjected to some hours of pre- and post-bombardment osmotic treatment with 0.5 M mannitol demonstrates that the pre-conditioning of the embryos and treatment with high osmotic strength are of paramount importance for successful transformation by particle bombardment. The fact that -given this pre-conditioning and osmotic adjustment- the use of MB3-1 led to the greatest number of transgenic plants subsequent to the widest range of bombardment conditions shows that this SCIM was indeed the best of the three tested for the production of transgenic wheat plants. The regeneration potential of explants and the calli formed from them is crucial for the success of genetic transformation of plants (Varshney and Altpeter, 2001; Altpeter *et al.*, 2005; Danilova *et al.*, 2007), and the superiority of MB3-1 may reside in its ability to preserve much of the 100 % regeneration capacity exhibited on this SCIM by non-bombarded embryos. Nevertheless, the adverse effects of bombardment were still clearly evident in the culture efficiencies shown for MB3-1 in Table 3 that were considerably lower than the 98.6 % observed without bombardment.

ML3-1 proved to be far less successful than MB3-1 in producing transgenic plants, despite the fact that it also gave rise to 100 % regeneration capacity and a higher yield of regenerated plants than MB3-1 upon *in vitro* embryo culture of non-bombarded embryos. This shows that the basal composition of the ML medium must be much less suitable than that of the MB medium for maintaining the integrity –and thus the regenerative potential– of the scutellum tissue. Nevertheless, Huber *et al.* (2002) obtained a transformation efficiency of 4.9 % with a German spring wheat cultivar on ML3-1 medium.

Rasco-Gaunt *et al.* (2001) found a wheat cultivar-specific increase in somatic embryogenesis and a marked improvement in post-bombardment shoot regeneration and stable transformation efficiency with a callus induction medium containing 9 % instead of 3 or 6 % sucrose. In contrast, our use of MB9-1 with 9 % maltose led to significantly poorer embryogenesis and plant regeneration with non-bombarded embryos than did the equivalent medium with 3 % maltose (MB3-1). MB9-1 was nevertheless included in the transformation experiments on the grounds that its higher osmotic strength might be of long-term benefit in stabilizing the water relations of

bombarded embryos. Even if this was the case, it was of no significant advantage for transformation, as this took place much more extensively on MB3-1 than on MB9-1. Nonetheless, even this "inferior" MB medium was distinctly more effective than ML3-1 as a SCIM for the production of transgenic plants.

The present investigation reveals that *in vitro* culture performance data gleaned from preliminary experiments carried out with non-bombarded embryos are of limited value for optimizing the ballistic transformation of spring wheat, since SCIMs resulting in good embryogenesis and plant regeneration with the untreated embryos may be less effective in promoting the repair of damage stemming from bombardment. How particular characteristics of a SCIM make it efficacious in producing transgenic plants subsequent to ballistic transformation cannot easily be deduced in advance, and a suitable medium must be identified experimentally on the basis of previous experience and findings with the particular genotype in question.

The importance of embryo pre-conditioning and osmotic protection

The necessity of both pre-bombardment culture of the excised immature embryos on SCIM and the presence of an osmotic agent prior to and following the bombardment for the production of transgenic plants indicates that both the medium and the osmoticum act in complement to enable the embryonic tissues to recover from bombardment injury.

In agreement with Klein and Jones (1999), we found that the time period of 4 days for which the embryos are incubated on SCIM prior to bombardment is critical for successful transformation. Other studies have reported the benefit of even longer periods of pre-culture (Takumi and Shimada, 1996; Vasil *et al.*, 1993). These studies did not, however, make use of the osmotic treatment that proved necessary in our experiments. A number of other investigators have also successfully employed pre- and post-bombardment osmotic treatment, mainly with 0.4 M sugar alcohols (Altpeter *et al.*, 1996; Jordan, 2000; Gil-Humanes *et al.*, 2011), although Huber *et al.* (2002) reported this treatment to be of no advantage. The 0.5 M mannitol we used as an osmoticum may induce cellular stress responses that prevent the initiation of an apoptotic reaction to particle penetration (Klein and Jones, 1999). In this vein, Pellegrineschi *et al.* (2000; 2002) successfully transferred freshly isolated embryos to MS medium containing 15% maltose directly prior to bombardment without any pre-culture, and Rasco-Gaunt *et al.* (2001) and Sparks and Jones (2009) had success in incubating embryos on a SCIM containing 9 % sucrose for 1-2 days prior to bombardment. These types of osmotic treatment were not successful in our experiments, and may thus be genotype-specific.

The role of physical bombardment parameters

Jordan (2000) observed better biolistic wheat transformation at an acceleration pressure of 1100 psi with 0.6 μm gold particles and target distances of 6 or 9 cm than with 1.0 μm particle size and 3 cm target distance, and Yao *et al.* (2007) achieved a higher rate of transformation with 0.6 μm than with 1.0 μm gold particles. We thus expected that 0.6 μm (rather than 1.0 μm) gold particles delivered at 900 (rather than 1350) psi and target distances of 9 or 12 cm (rather than 6) would bring the best results in our experiments. While the best culture and transformation efficiencies with both *Klein Brujo* and *Bobwhite* were indeed observed with a target distance of 9 cm, they were obtained with the larger 1.0 μm gold particles and higher 1350 psi acceleration pressure.

Souza Canada (2012) described transient transgenic GFP expression taking place mainly in the two surface layers of the scutella of bombarded immature wheat embryos, in agreement with findings of Huber (2002) and Fettig (1999). The penetration potential represented by the 1.0 μm particle size and the 1350 psi acceleration pressure was apparently most suitable for the deposition of the gold particles in the cells of these layers at a distance of 9 cm. This combination of physical parameter variants thus constituted the ballistic conditions giving rise to a most favorable balance between DNA delivery and cell damage with both wheat genotypes. The actual amount of the gold impacting on the target tissue will also influence this balance: the average of 250 μg of gold particles we deployed per bombardment falls within the range used by other investigators (Weeks *et al.*, 1993: 583 μg; Becker *et al.*, 1994: 29-126 μg; Altpeter *et al.*, 1996: 30-100 μg; Iser *et al.*, 1999: 250 μg; Rasco-Gaunt *et al.*, 2001: 60-120; Sparks and Jones, 2009: 58.8 μg; Gil-Humanes *et al.*, 2011: 58 μg).

All but one of the physical parameter combinations led to transformation with *Klein Brujo* to some extent, and the smaller gold particle size in combination with

each of the two shorter target distances and each of the two acceleration pressures resulted in the next best transformation efficiencies. These combinations of physical bombardment parameter values apparently also result in gold particle momentum sufficient to penetrate the scutellum tissue to an extent resulting in relatively good transformation. The failure of the combination of 0.6 μm gold particles, 900 psi acceleration pressure and 12 cm target distance to produce any transgenic plant may be due to failure in producing the necessary particle momentum for cell penetration.

Transformation efficiency

Even though higher transformation efficiency tended to reflect better culture efficiency in our work, there was no clear correlation between these two efficiencies. Iser *et al.* (1999) and Varshney and Altpeter (2001) found no parallelism between regeneration and transformation frequencies among different wheat genotypes, and the latter authors surmised that this reflects the action of independent genotypic factors. In our previous work (Souza Canada and Beck, 2013) we also found no correlation between embryogenic callus formation and regeneration capacity, the two factors that determine culture efficiency, which indicates that these two phenomena are also controlled by different genes or gene combinations. The transformation efficiencies that we observed with both *Klein Brujo* and *Bobwhite* varied considerably, not only in the mean values obtained with different bombardment parameter setting combinations, but also from replicate to replicate under any one particular combination of these settings. High levels of experiment-to-experiment variation in transformation efficiency have also been reported in others studies (Barro *et al.*, 1998; Rasco-Gaunt *et al.*, 2001). The best mean and single experiment transformation efficiencies of 10 % and 16.4 % that we obtained with *Klein Brujo* nevertheless indicate the potential of our methodology with this wheat genotype. The efficiency of biolistic transformation in wheat depends on the genotype (Takumi and Shimada, 1996; Iser *et al.*, 1999; Lazzeri and Jones, 2009). It was thus of interest to compare our transformation efficiency with *Klein Brujo* with that obtained with the model wheat genotype *Bobwhite* SH 98 26, which has been termed a super-transformable wheat line with transformation efficiencies consistently in excess of 70 % (Pellegrineschi *et al.*, 2002). Under our experimental conditions, the best mean and single experiment transformation efficiencies attained with *Bobwhite* were with 4.1 % and 6.9 %, i.e. less than 50 % of the corresponding values obtained with *Klein Brujo* and far lower than the efficiency reported by Pellegrineschi *et al.* (2002). The best transformation efficiencies obtained with *Bobwhite* by other investigators (Altpeter *et al.*, 1996; Nehra *et al.*, 1994; Becker *et al.*, 1994; Zhang *et al.*, 2000; Gil-Humanes *et al.*, 2011) have also not exceeded 7 %. Our present results indicate that *Klein Brujo* is a better wheat genotype than *Bobwhite* for transformation with our methodology.

Selection of transgenic plants

The total of 115 independent primary transgenic lines that we obtained in the present study was due in good part to the two-pronged screening procedure that was employed to identify and select transgenic callus and regenerating plantlets. The monitoring of both GFP expression and PPT resistance throughout the *in vitro* culture of the bombarded embryos permitted a ready identification of *gfp*-transgenics on the one hand and pointed to *bar*-transgenics in the absence of GFP fluorescence on the other. The repeated testing for GFP fluorescence during the *in vitro* culture additionally ensured that the loss of initially present GFP did not result from the loss of the transgene: if expression of the *gfp*-gene proved to be transient in some calli or regenerating plant material, these could still be "rescued" by testing for resistance to PPT indicative of the *bar*-gene. This strategy was invaluable in isolating as many transgenic lines as possible and thus for efficiently realizing the low transformation potential of wheat. Huber *et al.* (2002) also combined GFP screening with PPT selection during callus induction, but their strategy of further culturing only GFP-positive calli and shoots undoubtedly resulted in the loss of numerous transgenics. It is intriguing that numerous GFP-positive transgenics were first identified from apparently only PPT-resistant plantlets at the end of the shoot regeneration or rooting phases when they had become large enough to visually emerge from covering GFP-negative plant material.

We employed PPT for selection purposes at 4 mg/l in the present study, a concentration falling within the range used by other investigators (Rasco-Gaunt *et al.*, 2001: 3 mg/l; Pastori *et al.*, 2001: 4-5 mg/l; Pellegrineschi *et al.*, 2002: 5 mg/l; Huber *et al.*, 2002: 2-5 mg/l; Sparks and Jones, 2009: 2-6 mg/l). We commenced PPT treatment first at the completion of the callus induction phase to give the developing calli the opportunity to fully develop *bar*-

gene expression without premature selection pressure. This agrees with the finding of Rasco-Gaunt *et al.* (2001) that selection with the herbicide first during the later stages of plant regeneration, *i.e.*, after one or two rounds without PPT in regeneration medium, served to maximize the regeneration potential of the calli. In addition, Fadeev *et al.* (2006) reported higher transformation efficiencies when PPT was applied in the plant regeneration phase than when it was already included in the callus formation phase, and Altpeter *et al.* (1996) found that selection with PPT during the shoot regeneration phase rather than during prior selection cycles reduced the time required to obtain rooted transgenic plants.

The successful transfer of transgenic plantlets possessing a vigorous root system at the end of the shoot regeneration phase directly to soil eliminated the need for the time-consuming root regeneration phase.

Analysis of T0-plants

Although the transgenic T_0 plants we obtained from the selection procedure were phenotypically normal, some of them had fully or partially sterile ears. Sterility is a common phenomenon in transgenic cereals (Iser *et al.*, 1998). The fact that almost all of the 115 transgenic T_0 plants we obtained were shown by PCR to contain the *ipt*-gene shows that the rate of co-transformation of *ipt* and the selection genes *gfp* and/or *bar* from the co-integrate plasmid pGFPBAR we obtained with our transgenic T_0 plants was exceptional even in the light of the high values reported for other wheat genotypes. Our rates of 97.4 % co-transformation with *Klein Brujo* and 100 % with *Bobwhite* are markedly higher than those reported by Barro *et al.* (1997: 71.4 %), Leckband and Lörz (1998: 67 %), Stoger *et al.* (1998: 85 %) and Fettig and Hess (1999: 89 %).

The results of the RT-PCR and *Southern* blot analyses of the six *Klein Brujo* and two *Bobwhite* T_0 lines selected for further investigation corroborated the integration of the *ipt*-gene into the target genome of each transgenic plant. Whereas the *Southern* blots demonstrated the integration of at least one intact *ipt*-gene in all cases, the presence of additional positively reacting DNA fragments larger than the one representing the intact gene could represent undigested plasmid and/or truncations and rearrangements of the gene fragment. Rearranged multiple gene copies have been found several times in wheat transformation (Altpeter *et al.*, 1996; Becker *et al.*, 1994; Iser *et al.*, 1999; Nehra *et al.*, 1994; Weeks *et al.*, 1993; Fettig and Hess, 1999; Huber, 2002) and transgenes can undergo rearrangements prior to or during integration into the host genome (Altpeter *et al.*, 2005). The fact that all of the eight transgenic T_0 plants - even those not expressing GFP - evidenced PPT resistance signifies that the *bar*-gene had indeed been incorporated into the genomes of these plants and expressed.

Transgene inheritance

The results of the study of the inheritance of the introduced *gfp*-, *bar*- and *ipt*-genes up to the T_3 generation suggest that all of these genes were arranged in a linkage group and co-segregated as either a single dominant trait locus or as closely associated loci as described by Fettig (1999) and Altpeter *et al.* (2005), and that there was also at least one active copy for each gene. This would hold even if the transgenic plants contained more than one active copy of each of the genes, since multi-copy transgenic plants tend to exhibit all of the transgene copies at the same locus, irrespective of the number of transformation cassettes having been used (Jones, 2005; Altpeter *et al.*, 2005). The *ipt*-gene was present in almost all of the isolated transgenic T_0 lines and in the subsequent generations and was found to be inherited at a Mendelian ratio. On the other hand, a random and progressive loss of GFP expression and PPT resistance was observed in subsequent generations. One explanation for this may be the effect of silencing mechanisms on the *gfp*- and *bar*- transgenes, in which case the silencing of the *gfp*-gene would have been stronger. Anand *et al.* (2003) concluded that gene silencing in wheat is a random, progressive phenomenon that can be associated with a variety of mechanisms (Huber, 2002) including transcriptional and/or post-transcriptional gene silencing (Demeke *et al.*, 1999; Cannell *et al.*, 1999). The differential rate of expression of the *gfp*- and *bar*-genes we observed in our investigation may be rather associated with the different nature of the corresponding *CaMV35S*- and *Ubi1*-promoters, respectively, than with the transformation procedure or the transgene integration pattern. Chen *et al.* (1998; 1999) reported silencing of a *chitinase*-gene driven by the *CaMV35S*-promoter, but not of the similarly present *bar*-gene under the control of the *ubiquitin*-promoter. In other studies in which both a marker and a reporter gene were introduced into wheat, only one (Weeks *et al.*, 1993; Cannell *et al.*, 1999) or both (Fettig, 1999; Huber, 2002) of the genes were inactivated. Christensen *et al.* (1992) reported the *Ubi1*-promoter from maize to be the promoter inducing the highest

and most stable constitutive expression of the *bar*-gene in wheat, but Anand *et al.* (2003) reported that both the *Ubi1*- and *CaMV35S*-promoters are prone to random and progressive gene silencing in transgenic wheat, with the former being somewhat less susceptible to the silencing.

CONCLUSION

Our present investigation resulted in the production of a large number of transgenic lines of the Argentine spring wheat genotype *Klein Brujo* by bombardment of excised immature embryos that exhibited a very high rate of co-transformation of marker/selection genes and a candidate gene that were inherited over several generations with the characteristics of a single dominant trait locus. Our examination of the biological and physical parameters involved in the transformation itself and in the preservation of the original transgenes throughout the regeneration of transgenic plants pointed to an *in vitro* culture medium well suited to the establishment of regenerative transgenic callus, to the necessity of pre-bombardment culture and pre-and post-bombardment osmotic treatment of the embryos and to the advantages of bombardment with large gold particles and a high acceleration pressure for effecting transformation. Our use of a selection procedure based on two different co-transformed selection marker genes and our transfer of regenerants to soil as soon as possible enabled us to establish numerous transgenic plants *ex vitro* within a short time. The results point to the promise of *Klein Brujo* for large-scale transgenic applications, particularly with regard to the fact that this cultivar proved much more amenable to transformation than did the ostensibly super-transformable (Pellegrineschi *et al.*, 2002) wheat genotype *Bobwhite*.

The present study also points to the fact that the success of *in vitro* plant regeneration from non-bombarded wheat embryos cannot necessarily predict the success of transgenic plant regeneration from bombarded embryos, and that there is no really clear explanation of why a particular constellation of bombardment parameter settings leads to the best transformation successes. Finally, the progressive loss of transgene expression we observed in the present study is not yet fully understood and must be taken into account wherever the inheritance of artificially introduced transgenes is an issue.

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REGISTRO DEL CULTIVAR ANTONIO UNLPAM

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Antonio UNLPam es un cultivar de Triticale (x *Triticosecale* Wittmack), 2n=6x=42, inscripto en el Registro Nacional de Cultivares del Instituto Nacional de Semillas. Expte. INASE Nº 265.109, Resolución Nº 434/2012 del 03/12/2012. Fue obtenido por el Criadero A/1831 de la UN de La Pampa-Asociación Cooperadora, en colaboración con el Criadero A/1739 de la UN de Río Cuarto.

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Origen y proceso de selección

Deriva del cruzamiento original CIMMYT 150.83/SONNI:6-SWTY91.13-6FM-0FM. Fue seleccionado para uso forrajero de doble propósito (pasto y grano) a partir de introducciones recibidas en 1995 en la Facultad de Agronomía y Veterinaria de la UN Río Cuarto, formando parte del Ensayo Internacional de Triticales Forrajeros de Invierno (FWTCL) del CIMMYT. Se efectuó la selección individual en F_7 en Río Cuarto (Córdoba).

El comportamiento forrajero para doble propósito de varias líneas se probó en ECR durante 5 años en Santa Rosa, La Pampa y Río Cuarto. La selecta C95/46 se eligió por su muy buena producción de forraje invernal y grano frente a los testigos Genú-UNRC y Tizné-UNRC y Yagán INTA. Antonio UNLPam se fundó a partir de una planta individual de C95/46.

Considerando los resultados de producción de forraje (pasto y grano) se considera apto para emplear en la región pampeana semiárida-subhúmeda pampeana.

Producción de materia seca y grano

Presenta una rápida entrega de forraje en el primer corte, totalizando 48% de la producción, lo que facilita un rápido aprovechamiento del cultivo (Cuadro 1). La producción del segundo y tercer corte es de 35% y 16%, respectivamente. Presenta mayor entrega de forraje en el primer corte respecto a Yagán INTA. Supera significativamente a Tizné-UNRC y Yagán INTA en producción de grano.

Cuadro 1. Triticale Antonio UNLPam y testigos. Producción de materia seca y rendimiento de grano (kg/ha), 2009-2011 en Santa Rosa, La Pampa. Valores medios y significación.

Genotipo	Producción de Materia seca (kg/ha)				Rendimiento en Grano
	1er corte	2do corte	3er corte	Acumulado	
Antonio-UNLPam	1.429,5 a	1.039,2 a	496,1 a	2.964,8 a	2.923 a
Genú-UNRC	1.151,7 a	1.020,8 a	476,5 ab	2.649,0 a	2.600 ab
Tizné-UNRC	953,8 ab	892,1 ab	421,5 ab	2.267,4 ab	2.253 b
Yagán-INTA	790,8 c	981,4 ab	551,5 a	2.323,7 ab	2.325 b

Letras iguales en la misma columna indican diferencias no significativas.

Ciclo vegetativo

Hábito de crecimiento intermedio con porte juvenil semierecto, de vigoroso crecimiento inicial y con buen comportamiento sanitario durante todo el ciclo de aprovechamiento forrajero. En siembras para grano, el ciclo emergencia-floración plena es de 114,0±6 días y de 164,0±4 días a madurez de cosecha, dependiendo de las condiciones climáticas del año. El ciclo de emergencia a floración plena es 11, 7 y 6 días más corto que el de los testigos Genú-UNRC, Tizné-UNRC y Yagán INTA. La altura de planta a la madurez es de 84±15 cm según año y disponibilidad de agua durante el ciclo. La hoja inferior a la hoja bandera es más larga y ancha que la de los testigos. Presenta el hombro de la gluma inclinado y el relieve dorsal del cariopse convexo como características diferenciales respecto a los testigos.

La fecha de siembra más adecuada para la producción de forraje en la región subhúmeda-semiárida pampeana es en la primera quincena de marzo. La densidad de siembra se debe ajustar según calidad de semilla para obtener 200 plantas m^{-2} a la emergencia. La fecha de siembra más apropiada para producción de grano es la primera quincena del mes de junio.

Calidad comercial

Antonio-UNLPam presenta un Peso de 1000 granos = 33,8 g y Peso Hectolítrico = 65,6 kg/hL, como promedio de 3 años de evaluación (2009-2011). En 2011 se registraron los mayores valores de Peso de 1000 granos (38,5 g) y Peso Hectolítrico (67 kg/hL). Resulta apto como grano forrajero en la alimentación animal y tiene uso potencial en la alimentación humana para la elaboración de productos horneables que no requieran harinas leudantes y como materia prima para usos industriales como bioetanol.

Comportamiento ante factores adversos

Se ha comportado como tolerante a la sequía invernal y resistente a heladas durante el período juvenil pero como susceptible en floración. Presenta escasa severidad

Espigas

Gluma

e incidencia de roya de la hoja y manchas foliares que no comprometen la cantidad y calidad del forraje. No se ha registrado ataque de pulgón verde ni de otros fitoparásitos, así como tampoco vuelco por la ocurrencia de fuertes vientos primaverales ni arrebatamiento.

Pureza varietal

La pureza varietal se mantiene en el Campo Experimental de la Facultad de Agronomía de la UNLPam, sede del Criadero A/1831, mediante selección individual de una planta y posterior siembra de sus granos en surco y de allí a parcela en categoría de semilla prebásica. En los lotes de semilla fundación del Criadero de la Asociación Cooperativa de la Facultad de Agronomía se realizan periódicas observaciones durante el ciclo floración y madurez para la eliminación de las plantas fuera de tipo.

Granos

Vista dorsal

REGISTRO DE CULTIVAR ELENA-UNLPAM

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Elena-UNLPam es una variedad forrajera de maíz (*Zea mays* L.; 2n=2x=20), inscripta en el Registro Nacional de Cultivares del Instituto Nacional de Semillas. Expte. INASE Nº 494.798, Resolución INASE Nº 44/2014 del 19/03/2014. Fue obtenido en el Criadero A/1831 de la Universidad Nacional de La Pampa (UNLPam) -Asociación Cooperadora- Facultad de Agronomía.

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Origen de Elena UNLPam y proceso de selección

Esta variedad forrajera de maíz tuvo su origen en la cruza de maíz (*Zea mays* L.) con una especie relacionada (*Zea diploperennis* I. D. & G.), descubierta en México a mediados del siglo pasado y con interesantes características agronómicas, tales como numerosos macollos, resistencia a parásitos y adversidades climáticas, que despertaron el interés de introducirlas en el maíz.

A la cruza original le siguieron sucesivas retrocruzas de las plantas híbridas con maíz ¿con el propósito de incrementar el número y tamaño de mazorcas. Después de diez años de selección se originaron numerosas líneas experimentales con diferentes arquitecturas de planta forrajera y diferencias en la producción de materia seca digestible de planta completa y sus componentes morfológicos. Se seleccionaron las líneas más uniformes, generación tras generación, hasta llegar a la etapa de evaluación en ensayos comparativos.

Época de siembra, densidad óptima, regiones más adecuadas

Es una variedad de ciclo largo, de vigoroso crecimiento inicial, con numerosos macollos fértiles y con buen comportamiento sanitario durante el ciclo de cultivo. Por su característica de variedad de libre polinización presenta un amplio período de floración, lo que aumenta la probabilidad de fecundación y llenado de grano ante condiciones adversas de sequía y altas temperaturas en el periodo crítico.

La fecha más adecuada de siembra es a fines de octubre, cuando ha pasado el peligro de heladas tardías. De esta manera, llega a floración a fines de diciembre, por lo que esta etapa ocurre fuera del periodo de déficit hídrico del mes de enero.

La densidad de siembra es baja, a razón de 4,2 pl m^{-2}, que es cuando logra mayor producción de materia seca de planta completa; se han evidenciado diferencias entre separación de surco a 52 y 70 cm, a favor del último.

Se adaptaría a la zona norte de la provincia de La Pampa, donde el cultivo de maíz responde con mayor probabilidad de éxito. Se pretende, desde la Facultad de Agronomía de la UNLPam, abastecer con semilla convencional (no transgénica) y de menor precio a los pequeños productores de la zona.

Producción de Materia Seca

Elena-UNLPam tuvo un comportamiento similar al testigo en el primer corte de forrajimasa (30 días después de la emergencia) pero lo superó en 130% en la producción del rebrote (65 días posteriores a la emergencia) (Cuadro 1), acentuando su utilización forrajera para pastoreo directo.

Cuadro 1. Elena-UNLPam y testigo. Producción de Materia Seca (kg/ ha) del primer corte y rebrote en cultivo de secano en Santa Rosa, La Pampa 2007/2008. Valores medios y significación.

Genotipo	Producción de Materia seca (kg/ha)		
	Primer corte	Rebrote	Acumulada
Elena - UNLPam	740 a	1.152 a	1.892 a
DK 780 S	796 a	501 b	1.297 b

Letras iguales en la misma columna indican diferencias no significativas.

En la evaluación de la forrajimasa de planta completa con destino a silaje, la producción de Materia Seca de Elena-UNLPam fue similar a la de los testigos pero con mayor proporción de tallo y menor de grano (Cuadro 2).

Cuadro 2. Elena-UNLPam y testigos. Producción de Materia Seca (kg/ha) de planta completa y sus componentes botánicos al estado de 1/4 de avance de la línea de leche en el grano, en cultivo de secano en Santa Rosa, La Pampa; período 2006-2008. Valores medios y significación.

Genotipo	Producción de Materia seca (kg/ha)			
	Hoja	Tallo	Mazorca	Acumulado
Elena - UNLPam	2.504 a	9.586 a	3.471 d	15.326 a
DK 780 S	2.180 b	7.727 b	5.098 b	15.025 a
Tornado TD MAX	1.940 c	6.332 c	4.834 c	13.106 c
Pucará TDMAX	2.648 a	6.485 c	5.666 a	14.792 b

Letras iguales en la misma columna indican diferencias no significativas.

Elena-UNLPam presentó una producción de materia seca digestible (kg/ha) similar a la de un híbrido de sorgo forrajero en cultivo de secano durante 2009/2010 (Cuadro 3).

Cuadro 3. Elena-UNLPam y testigo. Producción de Materia Seca digestible (kg/ha) y de sus componentes botánicos para silaje en cultivo de secano en Santa Rosa, La Pampa, 2009/2010.Valores medios y significación.

Genotipo	Producción de Materia seca digestible (kg/ha)			
	Hoja	Tallo	Inflorescencia	Acumulado
Elena - UNLPam	2.078 a	4.617 b	1.817 a	8.512 a
Sorgo Nutritop	1.254 b	5.454 a	1.707 a	8.415 a

Letras iguales en la misma columna indican diferencias no significativas.

Comportamiento ante vuelco, heladas, sequía y golpe de sol

No se ha observado vuelco de la raíz por acción del viento aun cuando la morfología de la planta propendería a ello, por poseer varios macollos y mazorcas. Presenta muy buena recuperación a la acción del granizo en el periodo vegetativo pues produce nuevos macollos y es susceptible a las heladas tardías como los testigos utilizados. No se ha evidenciado vuelco a madurez debido a podredumbre o insectos barrenadores

Comportamiento ante Plagas

Se ha presentado susceptibilidad a *Helicoverpa zea, Diatraea saccharalis* y *Ustilago maydis* aunque los ataques no han comprometido ni la cantidad ni la calidad del forraje.

Pureza varietal

La pureza varietal se efectuará en el Campo Experimental de la Facultad de Agronomía de la UNLPam, mediante el procedimiento de libre polinización con selección de plantas individuales y cosecha en masa (CIMMYT, 1984).

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