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DIPLOID POTATO (*Solanum tuberosum* L.) AS A MODEL CROP TO STUDY TRANSGENE EXPRESSION

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Abstract: This paper presents a method of *Agrobacterium*-mediated transformation for two diploid breeding lines of potato, and gives a detailed analysis of reporter gene expression. In our lab, these lines were also used to obtain tetraploid somatic hybrids. We tested four newly prepared constructs based on the pGreen vector system containing the selection gene *npt*II or *bar* under the 35S or nos promoter. All these vectors carried *gus* under 35S. We also tested the pDM805 vector, with the *bar* and *gus* genes respectively under the Ubi1 and Act1 promoters, which are strong for monocots. The selection efficiency (about 17%) was highest in the stem and leaf explants after transformation with pGreen where *npt*II was under 35S. About half of the selected plants were confirmed via PCR and Southern blot analysis to be transgenic and, depending on the combination, 0 to 100% showed GUS expression. GUS expression was strongest in multi-copy transgenic plants where *gus* was under Act1. The same potato lines carrying multi-copy *bar* under Ubi1 were also highly resistant to the herbicide Basta. The suggestion of using *Agrobacterium*-mediated transformation of diploid lines of potato as a model crop is discussed herein.

Key words: Binary vectors, Gene expression, Genetic transformation, Polyploid

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Abbreviations used: BAP – 6-benzyladenine; GA_3 – gibberellic acid 3; GUS – β-glucuronidase; IAA – indole-3-acetic acid; NAA – 1-naphtalene acetic acid

INTRODUCTION

The cultivated potato, *Solanum tuberosum* L., is a tetraploid (2n = 4x = 48), vegetatively propagated species. As emphasized by Wolters and Visser [1], its genetic background is highly heterozygous compared to self-fertilizing, sexually propagated species. This could affect transgene expression/silencing. Diploid lines of potato are one of the main components of many breeding programs [2, 3]. Furthermore, wild diploid *Solanum* species contain valuable genes for potato improvement. It is feasible to manipulate potato ploidy in the range from monoploid to octoploid. The ploidy level can be scaled up through the functioning of 2n gametes and reduced through the use of *S. phureja* haploid inducers [4]. Alternatively, somatic hybridization can be employed [see review 5]. The diploid potato genome is 6 times larger than that of the model *Arabidopsis thaliana*, slightly smaller than that of tomato (*Lycopersicon esculentum*) and *Petunia hybrida*, and 5 times smaller than that of tobacco (*Nicotiana tabacum*). It is easier to perform molecular and genetic analyses on a 2n = 2x genome than on tetraploid potato. For some systems of research involving a transformation technique like transposon tagging, a diploid level of ploidy is required [6].

The techniques of tissue culture, *in vitro* plant regeneration and *Agrobacterium*-mediated transformation can easily be adapted to tetraploid cultivars [7-10] and to some diploid lines [11]. However, these techniques are genotype- and protocol-dependent. Potato genotypes are known for the large variation in their regeneration competence [12]. El-Kharbotly *et al.* [13] found and located a transformation competence factor in diploid potato lines. We expected that newly developed, efficient procedures of *in vitro* potato regeneration via somatic embryogenesis [14, 15] might facilitate *Agrobacterium*-mediated transformation. An additional advantage is that the somatic embryos mainly originate from single cells, and thereby avoid the problem of chimeric plant generation.

Somatic hybridization is another *in vitro* approach for the further improvement of commercial potato varieties. We developed techniques of protoplast culture, fusion and somatic hybrid generation from diploid breeding lines that were provided by breeders (http://www.ihar.edu.pl/gene_bank/potato/potato.php) and used in this and other our research [16, 17]. In vegetatively propagated crops, a selected hemizygous transgenic line might be used directly as a new variety or could be a component for a breeding program. The former implies that the transgene copy number (hemi-, homozygous) and/or ploidy level can be changed, affecting the expression of the transgene. An indication that ploidy level played an important role in transgene expression was already reported for GBSS transgenes in potato [18]. Transgene expression affected by ploidy and homozygosity was also reported for transgenic tobacco [19]. In potato, the accessibility of different techniques of ploidy manipulation enables the study of transgene expression against different genomic backgrounds. The lower ploidy level and simplicity of its modification makes the diploid lines a better experimental model than the commonly used tetraploid cultivars [20, 21].

The aims of this study were i) to develop a transformation system for diploid potato lines used in our lab for somatic hybridization; ii) to test new constructs in a pGreen vector system; and iii) to study GUS expression in different parts of transgenic plants growing *in vitro* and *in vivo*. Five of the lines were used to test regeneration ability, and the two best regenerating lines were transformed using *Agrobacterium*. A detailed analysis of the copy number and expression of the *gus* reporter gene was performed. The presented system is proposed as a model for the study of transgene expression in vegetatively propagated crop species.

MATERIALS AND METHODS

Plant material and *in vitro* culture

Leaf and stem explants of two *Solanum tuberosum* diploid lines, DG 88-596 (3C) and DG 82-330 (10J), were used in the experiments. The lines were provided by Prof. E. Zimnoch-Guzowska from Mlochow Research Centre, Plant Breeding and Acclimatization Institute. The donor plants were maintained as aseptic shoot cultures derived from stem cuttings, and rooted and cultured on 1/2 MS [22] (1/2X macroelements, microelements, Fe-EDTA) supplemented with 2% sucrose and 0.2% gelrite at 22ºC (day/night), with a 16-h photoperiod under cool white fluorescent light (35 μmol s^{-1} m^{-2}). 0.8- to 1-cm long stem explants were excised from the internodes of 3- to 4-week old plants and placed on the medium in a horizontal position. Leaf explants (c. 0.6 x 1 cm) were cut across the fully-developed leaves of the same *in vitro* grown plants and cultured with their adaxial surface in contact with the medium.

The basal medium for the *in vitro* culture contained Murashige and Skoog salts and vitamins (MS) plus 30 gl^{-1} sucrose and 2 gl^{-1} gelrite. Explants from 3C were cultured on the primary medium containing 0.2 mgl^{-1} NAA and 2 mgl^{-1} BAP, and after two weeks, subcultured on a medium with 5 mgl^{-1} GA_3 and 2 mgl^{-1} BAP [14]. The primary medium for the 10J explants contained 4 mgl^{-1} IAA and 0.04 mgl^{-1} BAP followed by a medium containing 2.5 mgl^{-1} zeatin rybosid plus 0.2 mgl^{-1} GA_3 [15]. The explants of both lines were subcultured for 4 weeks. Shoot development was done on MS medium without growth regulators. The cultivation conditions were the same as for the *in vitro* plants.

Transformation procedure

After two to three days of primary culture, explants were immersed in a suspension of *A. tumefaciens* and co-cultured for the next three days on the same medium. The subsequent culture was performed on the primary media containing 150 mg l^{-1} Timentin and a selection agent: kanamycin (100 mg l^{-1}) or phosphinothricin (2 mg l^{-1}). Appropriate selection was applied during the whole *in vitro* culture. Well-rooted plants were planted in the soil, and cultured to obtain microtubers or multiplied by continuous *in vitro* culture under selection.

Bacterial strains and vectors

We used two types of binary vector: the pGreen system [23, www.pgreen.ac.uk] and pDM805 [24]. pDM805 was provided by Dr. R. Brettell from CSIRO (Australia). It contained *bar* under the ubiquitin 1 promoter and *gus* under the actin 1 promoter. The schematic diagram of the vector's T-DNA showing the restriction sites is given in Fig. 1A. Four types of T-DNA construct were cloned into pGreen (Fig. 1B). The first contained a 35S::*npt*II selection cassette, the second nos::*npt*II, the third 35S::*bar*, and the fourth nos::*bar*. The reporter cassette, 35S::GUS, was cloned close to the RB of these vectors.

All the vectors were electroporated to *Agrobacterium* Agl1. The bacteria were cultured in MG/L liquid medium [25] supplemented with 50 mg l^{-1} rifampicin (*Agrobacterium* strain) and 50 mg l^{-1} kanamycin (pGreen) or 5 mgl^{-1} tetracycline (pDM805). After 2 days of culture at 28ºC, the bacteria were resuspended in MS medium at an OD_{600} between 0.6 and 1.0.

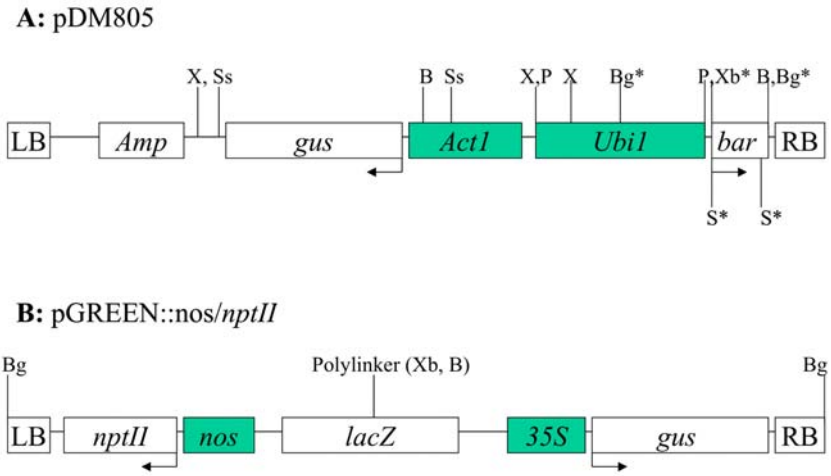

Fig. 1. The maps of the T-DNA regions of the pDM805 (A) and pGreen vectors (B). Restriction sites found in this study are marked with *. X – XhoI, Ss – SacI, B – BamHI, P – PstI, Bg – BglII, Xb – XbaI, S – SmaI. Abbreviations: Amp – ampicilin resistance gene, lacZ – complete lacZ gene.

PCR analysis and Southern hybridization

Genomic DNA was isolated from the young shoots of putative transgenic plants using the modified CTAB method of Murry and Thompson [26]. PCR amplification was carried out in a 25-µl reaction mixture containing 150 ng of template total DNA, 100 µM of each dNTP, 3 µM of each primer, 1 U Taq DNA polymerase (Promega), 2 mM $MgCl_2$ and 1x DNA polymerase buffer. A 700-bp *npt*II gene fragment was amplified with 5'-GAGGCTATTCGGCTATGACTG and 5'-ATCGGGAGCGGCGATACCGTA primers, and a 430-bp *bar* fragment was amplified with 5'-TCTGCACCATCGTCAACCACTACATC and

5'-CAGAAACCCACGTCATGCCAGTTC. Possible contamination of T_0 plants with *Agrobacterium* was checked with the *virA* specific primers, 5'-CTTCTTGAACTCGCCACCGC and 5'-AAGATCTGATCGATAATGAG, leading to the amplification of a 284-bp fragment. The amplification conditions were 94ºC for 1 min, 62ºC (*npt*II) or 68ºC (*bar*) or 55ºC (*virA*) for 1 min, and 72ºC for 2 min. The number of cycles was 35. The conditions were optimized for each pair of primers separately.

Ten micrograms of total DNA from T_0 plants was transformed with pDM805, digested with *Sac*I, *Xho*I, *Sma*I, *Xba*I and *Bgl*I and resolved by electrophoresis on 0.8% agarose gels. DNA was alkaline blotted under a vacuum onto positively charged nylon membranes (Roche). The transferred DNA was hybridized with PCR amplified and DIG-labeled fragments of *bar* (430 bp) or *gus* (606 bp). The probes were labeled using a PCR DIG probe synthesis kit (Roche). In both cases, 20 pg of plasmid DNA was used as a template. DNA from plants transformed with pGreen was digested with *Bgl*I, *Xba*I and *Bam*H1, and hybridized with the 606-bp *gus* probe. Prehybridization (1 h) and hybridization (16 h) were performed at 68ºC in a standard hybridization buffer (Roche). Detection was done using a non-radioactive method according to the manufacturer's protocol with CSPD as a chemiluminescent substrate. The light signals were detected on X-ray film.

Histochemical GUS assay and herbicide resistance

GUS expression was determined in the leaves, shoots, roots, buds (anthers and ovules), microtubers and tubers of T_0 plants (3-5 plants per line) using a histochemical GUS assay [27]. Tissue fragments were incubated overnight at 37ºC in a buffer containing 2 mM X-Gluc and 50 mM sodium phosphate buffer (pH 7.0). To test herbicide resistance, 3-week old plants of the X and Y lines (3 plants per line) growing in pots with soil were sprayed with basta solution containing 150 mg l^{-1} and 300 mg l^{-1} ammonium gluphosinate. The cultivation conditions were: 22ºC (day/night), with a 16-h photoperiod (150 μmol s^{-1} m^{-2} light). The effects were observed after two weeks.

RESULTS

Selection efficiency

We tested two selection systems using kanamycin or phosphinotrycin under the control of the nos, 35S or Ubi1 promoters (Tab. 1). The selection efficiency was expressed as the percentage of explants regenerating the plants under selection. The selection rate for better regenerating the 3C line (unpublished data) was highest after transformation with the 35S::*npt*II cassette and moderate with nos::*npt*II. There were no shoots selected on the medium with phosphinothricin after transformation with *bar* under 35S or nos in the pGreen system. There was selection of three transgenic shoots from stem explants observed with pDM805 where *bar* was under Ubi1. The selection for the second tested potato line, 10J,

was generally lower than for 3C. One to six plants were obtained from leaf explants transformed with *npt*II and *bar* under the control of nos or 35S, and the rates of resistance to kanamycin or phosphinothricin were from 1.8 to 3.7%. The selection efficiency of shoots regenerated from stem explants was from 0 to 12.2%. It was similar for both the 35S::*npt*II and nos::*npt*II cassettes, lower in the case of leaf explants, and much lower for stem explants transformed with *bar*.

Tab. 1. Selection efficiency of plants regenerated from leaf and stem explants of two diploid lines of potato after transformation with pDM805 and pGreen containing different selection cassettes.

Line/vector/constr.	Leaf explants			Stem explants		
	Number tested[1]	Selection effic.[2]	Number of plants	Number tested	Selection effic.[2]	Number of plants
3C/pGreen						
35S::nptII	24	16.7	8	46	17.4	44
nos::nptII	30	6.7	6	69	11.6	28
35S::bar	39	0	0	54	0	0
nos::bar	45	0	0	41	2.4	5
3C/pDM805						
Ubi1::bar	0	0	0	50	6.0	3
10J/pGreen						
35S::nptII	37	2.7	2	49	12.2	17
nos::nptII	54	3.7	6	60	11.7	27
35S::bar	45	2.2	5	59	3.4	5
nos::bar	56	1.8	1	58	0	0
Sum (range)	330	(0-16.7)	28	461	(0-17.4)	129

[1]pooled from two experiments, [2]the number of regenerating explants/number of all tested * 100

Tab. 2. The rates of PCR-positive and GUS-expressing transgenic plants selected on kanamycin or phosphinothricin.

Selection factor	Line/vector/constr.	Number of plants tested[1]	PCR positive (%)	GUS positive	
				Number	(%)
Kanamycin	3C/pG/35S::nptII	51	66	9	26
	3C/pG/nos::nptII	29	67	6	31
	10J/pG/35S::nptII	20	45	0	0
	10J/pG/nos::nptII	35	57	0	0
Sum (range)		135	(45 – 67)	15	(0-31)
Phosphinothricin	3C/pG/35S::bar	-	-	-	-
	3C/pG/nos::bar	5	0	0	0
	3C/pD/Ubi1::bar	3	100	3	100
	10J/pG/35S::bar	10	0	0	0
	10J/pG/nos::bar	1	0	0	0
Sum (range)		19	(0 – 100)	3	(0-100)

pG – pGreen; pC – pCambia, [1]PCR negative on the *Agrobacterium* gene

Expression vs. selection

Although all the selected shoots were rooted and cultured *in vitro* under continuous selection pressure, only some of them were PCR positive. For kanamycin, 45 to 65% of the selected plants were PCR positive, and for phosphinothricin, from 0 to 100% (Tab. 2). The rates of GUS-expressing transgenics among the selected PCR-positive plants were from 26 to 100%. The only vector combination where all (three) selected plants were PCR and GUS positive was pDM805. Detailed analysis of GUS expression was performed in different tissues of plants obtained after pGreen (#5, #8, #9, #32) and pDM805 (X, Y; Tab. 3 and Fig. 2).

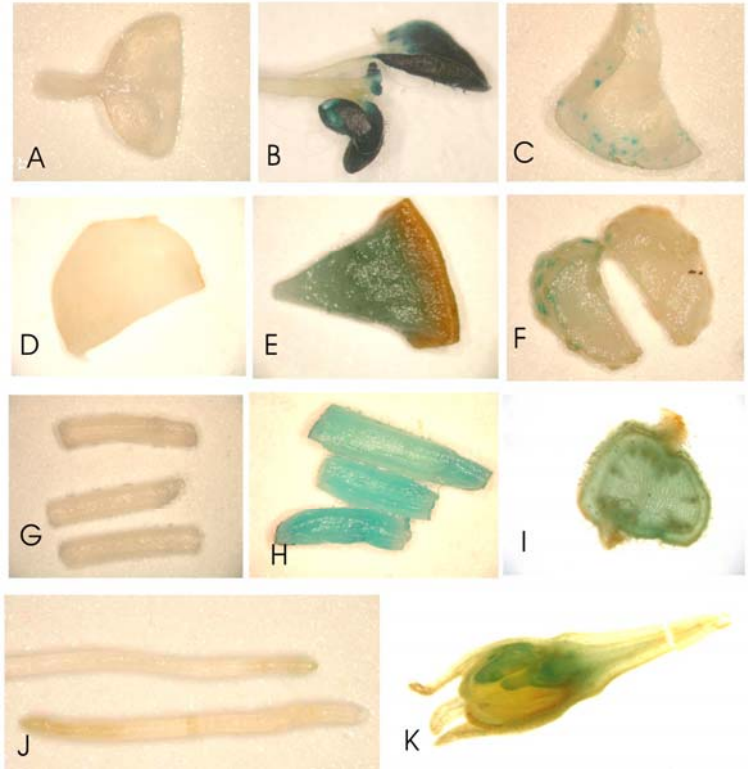

Fig. 2. Histochemical GUS staining in different organs. (A, D, G) Control, leaf, tuber and stem fragments from non-transgenic plants. (B) Shoot with apical meristem and young leaves (line Z). (C) Leaf fragment showing weak, point staining (line #9). (E, F) Tuber fragment (line Y) and cross-section through microtuber (line #8). (H, I) Stem fragments and cross-section (line Y). (J) Unstained roots of a transgenic plant. (K) Cross-section through a flower bud (line #9).

Expression vs. transgenic locus structure

Southern hybridization with different restriction enzymes allowed us to make detailed descriptions of transgenic loci and find the new restriction sites for the

plasmids (Fig. 1). According to the results, the Y plant contained at least four transgenic loci with one to several copies of T-DNA (Fig. 3A). This plant had the highest GUS activity (Fig. 2E, H, I). Furthermore, this phenotype was stable in different parts of the plant: leaves, shoots, microtubers, tubers and flower buds (Tab. 3). The X plant, transformed with the same vector, pDM805, contained about seven transgenic loci with one to several copies of T-DNA per locus. GUS

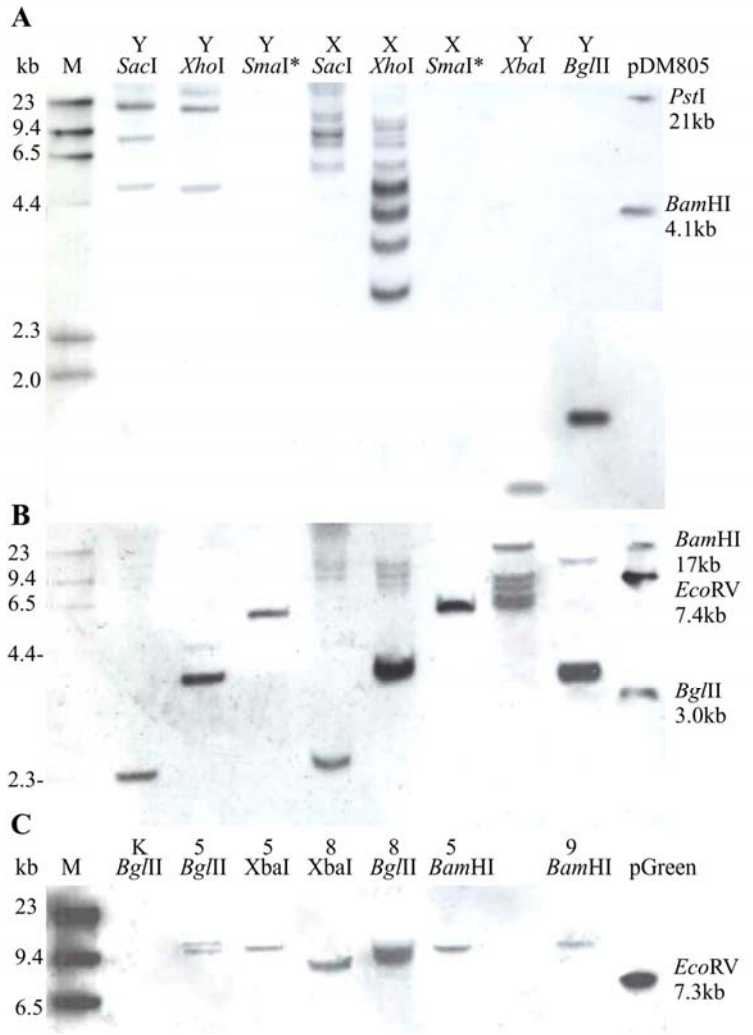

Fig. 3. Southern blot of transgenic potato lines transformed with pDM805 (A, B) and pGreen(nos::*npt*II) (C) and hybridized with *bar* pDM805 (A), *gus* pDM805 (B) and *gus* pGreen (C) probes. M – DIG labeled molecular marker; K – DNA from a control, non-transformed plant; * – c. 560-bp *bar* fragments (not shown).

expression was observed in almost all the tested parts of the plant. The third, Z line was also multicopy (not shown), expressing strong GUS activity (Fig. 2B). The transgenic loci of T-DNA in plants transformed with pGreen vectors were simple. The three tested GUS-expressing plants (#5, #8, #9) contained one or two loci with one to several copies of T-DNA. GUS expression in these plants was weak in *in vitro* leaves and microtubers and very weak in the leaves of soil-grown plants (Tab. 3; Fig. 2C, F).

Tab. 3. Histochemical analysis of GUS expression in transgenic lines of potato after one year of propagation in *in vitro* culture under selection pressure.

Line number	Root	Leaf		Shoot	Microtuber	Tuber	Flower buds
		in vitro	*in vivo*				
#5	-	+ spots	+ spots	-	-	-	++
#8	-	+ spots	-	-	+ & spots	n.t.	n.t.
#9	-	+ spots	+ spots	-	+ & spots	+spots	-
#32	-	+ spots	-	-	-	-	+
X	-	++	++	+++	n.t.	+++	+++
Y	-	++	+	+++	++	++	+++
Control (3C)	-	-	-	-	-	-	-

+ – weak; ++ – moderate; +++ – intense color; n.t. – not tested

Herbicide resistance

The X and Y plants were tested for Basta resistance with two doses of herbicide (Fig. 4). The control plants died after several days, while the X and Y plants grew well and produced tubers.

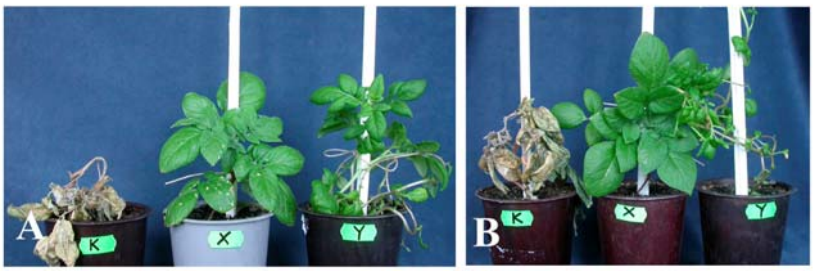

Fig. 4. Resistance to Basta herbicide after a two-week treatment with 150 mgl^{-1} (A) or 300 mgl^{-1} (B) ammonium gluphosinate. K – control, non-transformed plant.

DISCUSSION

Two types of binary vector in the Agl1 strain were used to transform diploid potato breeding lines. pDM805 was originally used for *Agrobacterium*-mediated barley transformation [24]. T-DNA contained selectable *bar* and reporter *gus* genes respectively under the control of Ubi1 and Act1, which are strong,

constitutive cereal promoters. The second vector system, pGreen, was developed by Hellens *et al.* [23]. This flexible and versatile small plasmid system allowed any arrangement of selectable marker and reporter genes and easy cloning of a suitable expression cassette. To compare selection efficiency on kanamycin or phosphinothricin, we cloned four cassettes containing *npt*II or *bar* under nos or 35S to T-DNA of pGreen carrying 35S::*gus*. Kanamycin selection was efficient, especially for stem explants. At least half of the selected plants were transgenic and 1/3 showed reporter gene expression. Phosphinothricin selection after transformation with pGreen, where *bar* was driven by the same promoters, was inefficient: it yielded several non-transgenic plants. The only phosphinothricin- and Basta-resistant plants were obtained after transformation using pDM805, where *bar* was under Ubi1, a constitutive promoter isolated from maize, which promoted high-level expression in monocotyledonous plants [28]. This promoter made it possible to select transgenic barley plants after *Agrobacterium*-mediated transformation [24]. It also promoted a stable, high-level expression of GUS in floral tissues and maturing grains of wheat, containing many copies of the transgene, produced by particle bombardment [29].

Different explants from tetraploid potato leaves [8], internodes [7, 9] and microtubers [30] were successfully transformed by *Agrobacterium*. The susceptibility of this species to *Agrobacterium*-mediated transformation allowed researchers to obtain relatively high efficiencies of transformation from 2.3% [11] to 31% [31] depending on the genotype, the features of the vector and strain, and the method itself. Only a few papers reported on the transformation of diploid lines [11]. In our lab, out of the five diploid lines tested, only two were successfully transformed. The highest transformation efficiencies of the two diploid lines were 11.6% for 3C and 6.7% for 10J. The procedures adopted by us for *in vitro* potato regeneration via somatic embryogenesis developed by JayaSree *et al.* [14] and Seabrook and Douglass [15] were also efficient for the 3C and 10J diploid potato lines, and proved to be appropriate for transformation.

The expression of *gus* under the constitutive Act1 promoter was strong, detected in all the organs of the plants and stable throughout *in vitro* and in-soil growth. The GUS expression driven by 35S was very weak in the leaves and microtubers, visible only in 1/3 of the transgenic plants. It would suggest that the strong monocot promoter Act1 was also strong for diploid potato, giving much better expression that 35S. However, strong and stable GUS-expressing and weak GUS-expressing plants also differed in the number of transgene copies integrated within the genome. The transgene copy number can be positively [32, 33] or negatively associated with transgene expression [34, 35]. Schubert *et al.* [36] characterized the correlation between the number of transgene copies and expression in *A. thaliana*. Expression of GUS driven by 35S was two-fold higher in homozygous plants harboring two copies than in hemizygous plants with a single copy. In the lines containing more than two copies of the transgene, transcript level-mediated silencing was detected. Diploid potato lines containing one to three copies of *gus* driven by 35S showed very weak expression in the

selected organs. The high and stable expression of selection and reporter transgenes in diploid potato was associated with the presence of more than four transgene copies. There was also a positive association between the transgene copy number and GUS in hemizygous (T_0) barley plants transformed with the same construction [24].

Two different groups of transgenic lines, a high and low copy number, were obtained after transformation with two binary vectors containing different selection cassettes carrying the same *Agrobacterium* strain. This might suggest that some binary vectors had the tendency to give higher copy T-DNA integration than others. Copy number in barley plants transformed with pDM805 was generally higher [24] than in tobacco plants transformed with pGreen [23]. Additional virulence genes in the pGreen binary system significantly altered plant transformation efficiency in rice but had no effect on transgene copy number, percentage of expressing lines or expression level [37]. However, the lack of selection of phosphinothricin-resistant plants in combinations transformed with pGreen, where *bar* was also under the 35S promoter, which is strong for dicotyledonous plants, might suggest a positive correlation of copy number with the level of resistance.

We have presented a relatively easy method for crop plant transformation. The diploid genome is small enough for detailed molecular analyses. Easy manipulations of ploidy level make this system suitable for studying the relationship of transgene expression to different ploidy level. The method might be used to test different vectors and the selection or expression of new constructs in vegetatively propagated plants.

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