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 nptII 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 nptII 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; GA3 – 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 22°C (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 g l⁻¹ sucrose and 2 g l⁻¹ gelrite. Explants from 3C were cultured on the primary medium containing 0.2 mg l⁻¹ NAA and 2 mg l⁻¹ BAP, and after two weeks, subcultured on a medium with 5 mg l⁻¹ GA₃ and 2 mg l⁻¹ BAP [14]. The primary medium for the 10J explants contained 4 mg l⁻¹ IAA and 0.04 mg l⁻¹ BAP followed by a medium containing 2.5 mg l⁻¹ zeatin ribosid plus 0.2 mg l⁻¹ GA₃ [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::nptII selection cassette, the second nos::nptII, 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 mg l⁻¹ tetracycline (pDM805). After 2 days of culture at 28°C, the bacteria were resuspended in MS medium at an OD₆₆₀ between 0.6 and 1.0.

A: pDM805

![Diagram of pDM805 vector]

B: pGREEN::nos/nptII

![Diagram of pGreen vector]

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 – Xhol, Ss – SacI, B – BamHI, P – PstI, Bg – BglII, Xb – XbaI, S – SmaI. Abbreviations: Amp – ampicillin 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 MgCl₂ and 1x DNA polymerase buffer. A 700-bp nptII gene fragment was amplified with 5’-GAGGCTATTCGGCTATGACTG and 5’-ATCGGGAGCGGCGATACCGTA primers, and a 430-bp bar fragment was amplified with 5’-TCTGCACCATCGTCAACCACACTACATC and
5'-CAGAAACCCACGTCATGCCAGTTC. Possible contamination of T₀ plants with *Agrobacterium* was checked with the *virA* specific primers, 5'-CTTCTTGAACTGCCACCGC and 5'-AAGATCTGATCGATAATGAG, leading to the amplification of a 284-bp fragment. The amplification conditions were 94°C for 1 min, 62°C (*nptII*) or 68°C (*bar*) or 55°C (*virA*) for 1 min, and 72°C 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 T₀ plants was transformed with pDM805, digested with *SacI*, *XhoI*, *SmaI*, *XbaI* and *BglI* 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 *BglI*, *XbaI* and *BamHI*, and hybridized with the 606-bp *gus* probe. Prehybridization (1 h) and hybridization (16 h) were performed at 68°C 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 T₀ plants (3-5 plants per line) using a histochemical GUS assay [27]. Tissue fragments were incubated overnight at 37°C 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: 22°C (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::*nptII* cassette and moderate with nos::*nptII*. 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 nptII 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::nptII and nos::nptII 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 | Selection effic. | Number of plants | Selection effic. | Number of plants |
| 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              |
| 35S::bar            | 39            | 2.7            | 2               | 49             | 12.2           | 17             |
| nos::bar            | 54            | 3.7            | 6               | 60             | 17.2           | 27             |
| nos::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            |

1pooled from two experiments, 2the 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 | 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, 1PCR 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.](image)

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::nptII) (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 \textit{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 \textit{in vitro} culture under selection pressure.

| Line number | Root | Leaf | Shoot | Microtuber | Tuber | Flower buds |
|-------------|------|------|-------|------------|-------|-------------|
| #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 mg\textsuperscript{l} (A) or 300 mg\textsuperscript{l} (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 \textit{Agrobacterium}-mediated barley transformation [24]. T-DNA contained selectable \textit{bar} and reporter \textit{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 nptII 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 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 (T₀) 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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