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Publisher's version / Version de l'éditeur:

<https://doi.org/10.1007/s11240-022-02434-x>

Plant Cell, Tissue and Organ Culture (PCTOC), 152, 3, pp. 605-618, 2023-01-25

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A protocol for *Agrobacterium*-mediated genetic transformation of *Lens culinaris* Medik (lentil)

Patricia L. Polowick¹ · Wei Yan¹

Received: 15 July 2022 / Accepted: 11 December 2022 / Published online: 25 January 2023

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Abstract

A reliable protocol for *Agrobacterium*-mediated genetic transformation of *Lens culinaris* Medik (lentil) was developed. Using cultivar Laird, the protocol yielded rooted shoots from an average of 6.8 independent events per hundred seeds. The protocol utilized longitudinal slices of embryo axes from imbibed mature seed as a starting explant and a plasmid containing a β -glucuronidase:neomycin phosphotransferase (*gus:nptII*) fusion gene in *Agrobacterium* strain EHA105. A series of four media, each with appropriate levels of kanamycin selection were identified and other factors tested included the optical density of the *Agrobacterium* suspension, and type and concentration of plant growth regulators. The expression of the *gus* reporter gene was visualized through histochemical staining, and further molecular analysis through PCR, qPCR, ddPCR and Southern hybridization confirmed transformation and provided copy number. The inserted genes were inherited into the T₁ generation and chimaeras were not identified. The time from co-cultivation to the planting of rooted shoots ranged from 4 to 7 months, as transgenic clusters continue to produce additional clonal shoots.

Key message

A protocol for *Agrobacterium*-mediated genetic transformation in with a higher frequency than previous reports, multiple shoots per transgenic event, consistent rooting, minimal escapes, no evidence of chimaeras and confirmed inheritance.

Keywords Lentil · *Agrobacterium* · Genetic transformation

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
Gus	β -Glucuronidase
ddPCR	Droplet digital PCR
GMO	Genetically modified organisms
CRISPR	Clustered regularly interspaced short palindromic repeats
<i>nptII</i>	Neomycin phosphotransferase
OD	Optical density
BAP	Benzylaminopurine
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
X-Gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt

Communicated by Sergio J. Ochatt.

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Introduction

Lentil (*Lens culinaris* Medik) is a grain legume food crop that was domesticated in the near east, as early as neolithic times, becoming a staple crop more than 7000 years ago (Muehlbauer and McPhee 2005). As with other legumes, it is a beneficial crop to include in rotation (Warne et al. 2019) due to its ability to fix nitrogen through a symbiotic relationship with soil *Rhizobium* (Maróti and Kondorosi 2014). Lentil is also well adapted to low moisture conditions (Warne et al. 2019).

Lentils are nutritious, with 21–31% protein, depending upon the cultivar, and they are also rich in dietary fibre, vitamins, as well as zinc and iron (Romano et al. 2021). They are fast-cooking relative to the other grain legumes, and popular in the Middle East, North Africa and South Asia (Muehlbauer and McPhee 2005). Worldwide, over 6 million tonnes of lentil are produced every year, with Canada as the largest producer with > 3 million tonnes, followed by India with > 1 million tonnes (AtlasBig 2018–2020). Due to the upsurge in world population as well as interest in a plant-based diet, there is and

will continue to be an increased demand for the grain legumes due to their high protein content.

In order to satisfy the increasing demand for legumes while contending with population growth and climate change, including the possibilities of a shrinking arable land base, genetic improvements are required to increase overall yield and seed quality. Anticipated targets of interest relate to responses to both biotic and abiotic stresses such as disease, drought resistance and competitiveness with weeds. Genetic improvements are facilitated by marker assisted breeding and recent release of extensive genetic sequencing of this crop (Ramsay et al. 2021). Genetic transformation resulting in GMO crops has not been particularly viable due to general lack of acceptance in many countries that regularly consume legumes or use them as animal feed. However, CRISPR-based gene editing of crops provides an option that is gaining acceptance by regulatory bodies (Turnbull et al. 2021) as the changes made are generally small deletions in native genes; any inserted DNA that is part of the CRISPR machinery can be removed through crossing. Protocols to incorporate the CRISPR components and to regenerate complete plants are prerequisites for benefiting from the opportunities presented by gene editing, as well as to facilitate validation of gene functioning through the production of modified plants.

Another factor influencing the production of transgenic cultivars is the paucity of reports of successful lentil genetic transformation that result in the regeneration of a complete plant (Chopra et al. 2012). Rooting in vitro presents a bottleneck occasionally bypassed by grafting onto rootstock or continued growth and flowering in vitro (Gulati et al. 2002; Akcay et al. 2009; Das et al. 2019). A consistently low frequency of success, as seen with most grain legumes, ranged from 0.35% (Das et al. 2019) to a high of 3.1–6% (Gulati et al. 2002), although the means of calculation differed and, on occasion, included multiple clonal shoots from one explant and/or escapes.

The goal of this study was to develop a reliable transformation protocol for lentil to be used for gene function validation and for gene editing and is based on previous experience with other grain legumes, including *Pisum sativum* L. (pea; Polowick et al., 2000), *Cicer arietinum* L. (chickpea; Polowick et al. 2004) and *Lupinus mutabilis* Sweet. (tarwi; Polowick et al., 2014).

Materials and methods

Seed source and *Agrobacterium* strain

Seed of *Lens culinaris* Medik. cv. Laird and CDC Milestone were obtained from the Plant Gene Resource of Canada (Agriculture and Agri-food Canada, Saskatoon, SK).

The *Agrobacterium* culture LBG66 used in this study was described previously (Polowick et al. 2000). LBG66 consists of plasmid pPBI3010, which contains a fusion gene (*gus:nptII*) conferring both β -glucuronidase (*gus*) and neomycin phosphotransferase (*nptII*) functions (Datla et al. 1991) with a constitutive 35S35SAMV promoter (Datla et al. 1993), a nopaline synthase (*nos*) terminator and an intron (Vancanneyt et al. 1990), electroporated into the disarmed *Agrobacterium* strain EHA105 (Hood et al. 1993).

Preparation of *Agrobacterium*

Overnight *Agrobacterium* cultures, grown in 2YT medium with antibiotics (30 mg/L rifampicin, 25 mg/L gentamycin), were harvested by brief centrifugation and resuspension in B5 medium (Gamborg et al. 1968) without antibiotics. For co-cultivation, the *Agrobacterium* suspension was adjusted to a final OD of 0.1 or 0.2 at A_{660} (600 λ) with B5 basal medium.

Preparation of explants

Seeds (100 per experiment) were rinsed in 70% (v/v) ethanol then immersed in 10% of commercial bleach solution (Clorox™), equivalent to 0.525% (w/v) NaOCl, for 6 min. The seeds were rinsed thoroughly with sterile distilled water and imbibed on sterile wet filter paper in deep Petri dishes (100 × 25 mm) at room temperature (23 °C) in the dark for approximately 16 h (overnight). Fully imbibed seeds with intact seed coats (i.e., radicle not emerged) were used for explant isolation. The explants used in this study included cotyledons with or without cotyledonary nodes wounded with by a needle, and sliced embryo axes.

To isolate the embryo, the seed coat was removed and one cotyledon as well as the distal half of the radicle was excised. For embryo slices, the remaining cotyledon with the embryo axis still attached was secured with forceps and the embryo axis was cut into longitudinal sections (2–3 per seed) with a blade that had been dipped into the *Agrobacterium* suspension. Alternately, an OD of 0.1 was used to sonicate embryo slices in *Agrobacterium* for 2, 5, and 10 s. Whole embryos were sonicated for 10 or 30 s in OD 0.2 of *Agrobacterium*.

With an OD of 0.1, the tested methods of *Agrobacterium* application included incubation for 20 min with shaking, or deposition of droplets to individual explants (5 μ l in the case of slices).

Co-cultivation

The explants were plated onto co-cultivation medium, in 15 × 60 mm Petri plates. The embryo slices were placed on sterile filter paper positioned on top of the co-cultivation

medium, with slices from 10 seeds, or 10 cotyledonary nodes per Petri dish. Except where otherwise noted, approximately 100 seeds were used for each slicing experiment. For cotyledons, and cotyledons with wounded nodes, the explants were plated equally on their adaxial or abaxial surfaces, with 50 per experiment. Each experiment was repeated at least three times, unless the results from the first two trials indicated that this was not worthwhile.

Three different basal media were tested for the co-cultivation period. These included MS basal medium with B5 vitamins (Murashige and Skoog, 1962; Gamborg et al. 1968), B5 with B5 vitamins (Gamborg et al. 1968) and B5h, a minimal medium with 2,4-dichlorophenoxyacetic acid (2,4-D; 1 mg/l) and kinetin (0.2 mg/l), as previously described for pea (Polowick et al. 2000). This and all subsequent media were supplemented with 3% sucrose, adjusted to a pH of 5.8, solidified with 0.8% agar and sterilized by autoclave. Acetosyringone (100 μ M) was a consistent additive to the co-cultivation medium. Other additives tested included lipoic acid, which was tested at 5, 10 and 50 μ M. All plant growth regulators and additives were filter sterilized and added after autoclaving. The explants were incubated at 25 °C with a 16 h photoperiod of LED light at 40 μ mol m⁻² s⁻¹ for co-cultivation periods of 3 or 4 days, with random samples of 10 explants collected for histochemical staining for *gus* gene expression.

Shoot induction

The explants were transferred to shoot induction medium (MS macro- and micronutrients (Murashige and Skoog, 1962), and B5 vitamins (Gamborg et al. 1968) in 15 × 60 mm Petri plates. Benzylaminopurine (BAP) was tested at concentrations of 1, 2 and 3 mg/L. A preliminary test also included lipoic acid at 5, 10 and 50 μ M. To control *Agrobacterium* overgrowth, this and all subsequent media contained the antibiotic mixture Timentin® (200 mg/l), filter sterilized and added to cooled autoclaved media. The explants were incubated at 25 °C with a 16 h photoperiod at 40 μ mol m⁻² s⁻¹ with dimmable T5 841 High Output Fluorescence bulbs (4100 K, Philips, Andover, MA, USA) lighting. A preliminary kill curve revealed that most explants produced shoots in the presence of 20 mg/l kanamycin during the shoot initiation phase, but the majority were dead with 40 mg/l kanamycin. Based on these results, explants were exposed to 25 or 30 mg/l kanamycin for subsequent tests. The explants remained on this medium for 3 weeks.

Shoot development and elongation

Surviving explants were transferred to MS basal medium with B5 vitamins and BAP (1 mg/l), in 25 × 100 mm Petri dishes. The selection chemical kanamycin was added to

the medium over a range of concentrations of 30, 40, 50 and 60 mg/l. Surviving explants were transferred to fresh shoot elongation medium every 14 days (or more often in the case of *Agrobacterium* overgrowth), with kanamycin increased after each round to a maximum of 60 mg/l for at least 4 rounds.

Rooting

Elongated shoots > 1.5 cm and with at least one leaf node below the apical meristem were transferred to rooting medium in deep (25 × 100 mm) Petri dishes. Media tested included half-strength B5 basal medium (half-strength B5 vitamins) supplemented with naphthalene acetic acid (NAA; 0.2 mg/l), and indolebutyric acid (IBA; 0.2 mg/l), alone or in combination. To promote induction of rooting, kanamycin was not used at this stage.

Once roots were well established, and the shoots were over 2.5 cm in height, young putative transformants were transferred to commercial soilless mixture (Sunshine No. 4, Sun Gro Horticulture, Bellevue, Wash.) in 13 cm pots and grown in a growth chamber at 22/18 °C (day/night) with a 16 h photoperiod provided by lighting supplied by T5 fluorescent 835 (3500 k) and with a far red LED (Ray Series, PfrSpec™, Fluence by Osram; Austin, TX, USA) to encourage flowering. The plants were initially covered with a clear plastic cup until well established. After one week, the covering was removed gradually over a period of 3 or more days, depending upon the perceived vigour of the plantlet. The plants were fertilized at each watering with 20–20–20 fertilizer (0.2 g/l; Plant Products Co., Bramalea, ON, Canada) before flowering and 15–30–15 (0.27 g/l; Plant Products Co., Bramalea, ON, Canada) after flowering initiated.

Analysis

For a visual identification of *gus* activity, explants or plant material were incubated at 37 °C for 24 h in a 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid cyclohexylammonium salt (X-Gluc) solution as described by Jefferson (1987).

For PCR analysis, a crude genomic DNA was extracted using a protocol modified from Edwards et al. (1991). Primers used were for sequences in *nptII* (Table 1), with an expected band size of 479 bp. Using an AmpliTaq Gold® 360 Master Mix (Applied Biosystems) kit, the PCR conditions included a start at 95 °C for 10 min, 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 7 min (one cycle). The amplified

Table 1 Sequence summary of primers, probes and reference genes for analysis of lentil putative transformants

Primers & Probes	Sequence (5'–3')
NPTII forward	ATCTCCTGTCATCTCACC
NPTII reverse	AAGAAGGCGATAGAAGGC
GUS forward	GGAAAGCGCGTTACAAGAAAG
GUS reverse	AGA CGT TGC CCG CAT AAT
GUS probe	56-FAM/AAC GAT CAG /ZEN/ TTC GCC GAT GCA GAT /3IABkFQ/
Reference gene forward	GTCAACTCGTCCGGTTGTTA
Reference gene reverse	CACGGGTCGATTATTCTGTCT
Reference gene probe	5HEX/TGG TTG AAA /ZEN/ CGC AGG AGA AAG GGA /3IABkFQ/

products were separated by electrophoresis on 1% agarose gels.

Copy number analysis

For copy number analysis, three different approaches including quantitative PCR (qPCR), droplet digital PCR (ddPCR), and non-radioactive Southern hybridization were used to determine the best method for this material.

For qPCR analysis, a Qiagen DNAeasy Plant Mini Kit (Qiagen, Germantown, MD, USA) was used to extract genomic DNA from 20 mg of freeze dried young leaves of putative transgenic plants and controls. The amplification was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems; Waltham, MA, USA). The qPCR reaction mixtures were carried out in a 10- μ L volume containing 2 \times PowerUpTM SYBRTM Green Master Mix (Applied Biosystems; Waltham, MA, USA), 25 ng of genomic DNA, and 0.5 μ M primers for the gus gene and a section of a single copy, 1463 bp reference sequence (*Lens culinaris* contig01460.Lecu mRNA sequence; <https://www.ncbi.nlm.nih.gov/nuccore/JI847742.1/>), identified in cv. CDC Redberry and confirmed in cv. Laird, with expected 98 and 102 bp products produced, respectively. Primer sequences used are listed in Table 1. The qPCR conditions included a 2 min start at 50 °C, with 2 min at 95 °C and 40 cycles of amplification for 15 s at 95 °C, 1 min at 60 °C, ending with melting curve analysis. Using DNA from a non-transformed plantlet as a control sample and the single copy homozygous sequence as a reference, the delta-delta CT ($\Delta\Delta$ Ct) method (Livak and Schmittgen 2001) was adopted to derive the relative expression value for further analysis. Two to three technical replicates were performed for each plant.

For Southern hybridization analysis, the genomic DNA extracted for the qPCR was used. The DNA (10 μ g) was digested with *Hind*III at 37 °C overnight, separated on a

0.9% agarose gel and transferred by capillary blotting (Sawada et al. 1995) onto positively charged nylon membrane (Roche; Catalogue # 1–209-299). Using a DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche, Mannheim, Germany), the cut DNA was probed with an 1,800-bp BamHI/SstI fragment cut from the plasmid pBI121 (Jefferson 1987), encompassing the entire *gus* gene.

For ddPCR analysis of copy number, DNA extraction was as per qPCR. A section of the homozygous, single copy 1463 bp sequence identified for qPCR was used as a reference. All primer/probe sequences are listed in Table 1. 1 μ g of genomic DNA was digested with 10 μ l of *Hind*III-HF (New England Biolab; Ipswich, MA, USA) at 37 °C for 1 h. A ddPCR™ Supermix for Probes (BioRad Laboratories; Hercules, CA, USA) was used for all reactions. Each 20 μ l reaction contained 10 μ l 2x ddPCR supermix for probes 1 μ l 20xGUS primers/probe mix (final concentration of 900 nM/250 nM), 1 μ l 20xReference primers/probe mix (final concentration of 900 nM/250 nM), 1 μ l *Hind*III digested lentil DNA (25 ng/ μ l) and 7 μ l ddH₂O. The PCR amplification program included an initial denaturation at 95 °C (10 min) followed by 40 \times (94 °C for 30 s; 56 °C for 1 min, with a ramping rate of -2 °C/sec), and ending with 98 °C for 10 min using a T100 PCR Thermal Cycler (Bio-Rad Laboratories). Using a QX200 instrument (Bio-Rad Laboratories; Hercules, CA, USA), the reactions and droplets were analyzed according to manufacturer's instructions. There were three replicates per putative transgenic event, including a negative control. The data was analyzed with QuantaSoft software (Bio-Rad Laboratories, Hercules, CA, USA).

Inheritance

The T₁ seed (20 per line) of 11 lines was planted for analysis to confirm inheritance and to determine the ratio of positive to negative plants in the next generation, with potential reference to chimaeras. The number of plants varied based on seed germination. The testing was via *gus* staining of the entire seedling or shoot material and/or PCR analysis. The results for single copy lines were further analyzed by Chi-square analysis.

Results

An *Agrobacterium*-mediated method for the production of transgenic lentil (*Len culinaris* Medik) was developed using a vector with a constitutively-expressed *gus* reporter gene and *nptII* for chemical selection using the antibiotic kanamycin. Different explants, including cotyledons, cotyledons with attached and wounded nodes and longitudinal slices through the embryo axis, were tested. A range

of media were evaluated for each developmental stage, including co-cultivation, shoot induction, shoot elongation and rooting. These media ranged from a few simple salts to complete basal media, both with and without plant growth regulators and acetosyringone. The kanamycin concentration was influenced by a kill curve on untransformed tissue at each state. Two cultivars of lentil were used in the experiments, starting and largely focusing on cv. Laird, with preliminary testing on CDC Milestone. Histochemical staining of *gus* activity and molecular analysis confirmed transformation, indicated copy number and tracked inheritance into the T₁ generation.

Examples of results from various tests are presented with those representing the best conditions, as identified within the current, study are visualized in Figs. 3 and 4.

Co-cultivation

Cotyledons with abaxial and adaxial sides up, cotyledon with wounded nodes attached, in both orientations (Fig. 1a, b, respectively) and longitudinal slices through the shoot portion of the embryo axis (Figs. 1c, 3a) were each co-cultivated with *Agrobacterium*. At the end of co-cultivation, faint X-Gluc histochemical staining for *gus* gene activity was observed in the nodal region of cotyledonary explants with nodes, that was more distinct on explants with the cotyledon

placed on its abaxial surface. In contrast, distinct staining was visible on the incubated slices of the embryonic axis (Figs. 1f, 3b).

Three co-cultivation media namely Gamborg's B5 basal salts and vitamins, a minimal medium B5h with added and MS basal salts (B5 vitamins) were tested with the sliced material (Fig. 1c, d, e). Based on X-Gluc histochemical staining at the end of co-cultivation, the explants on minimal B5h (Fig. 1f) medium and those on MS medium exhibited minimal lower transient expression of the *gus* gene, while stronger expression was observed on Gamborg's B5 (Fig. 1f; Fig. 3b).

Transient *gus* expression, as visualized with X-Gluc staining, was stronger after a 3 day period of co-cultivation than after 4 days (Fig. 1g, h, respectively); the longer co-cultivation period also consistently resulted in *Agrobacterium* overgrowth on 85% of the plates, reducing the sample size for regeneration.

The use of sonication did not provide a benefit to transformation efficiency. The transient expression of the *gus* gene was not as high in the sliced explants it was with the other methods of *Agrobacterium* exposure, with the expression decreasing with increased duration of sonication (Fig. 2 a,b,c). In addition, *Agrobacterium* overgrowth was an issue with this treatment.

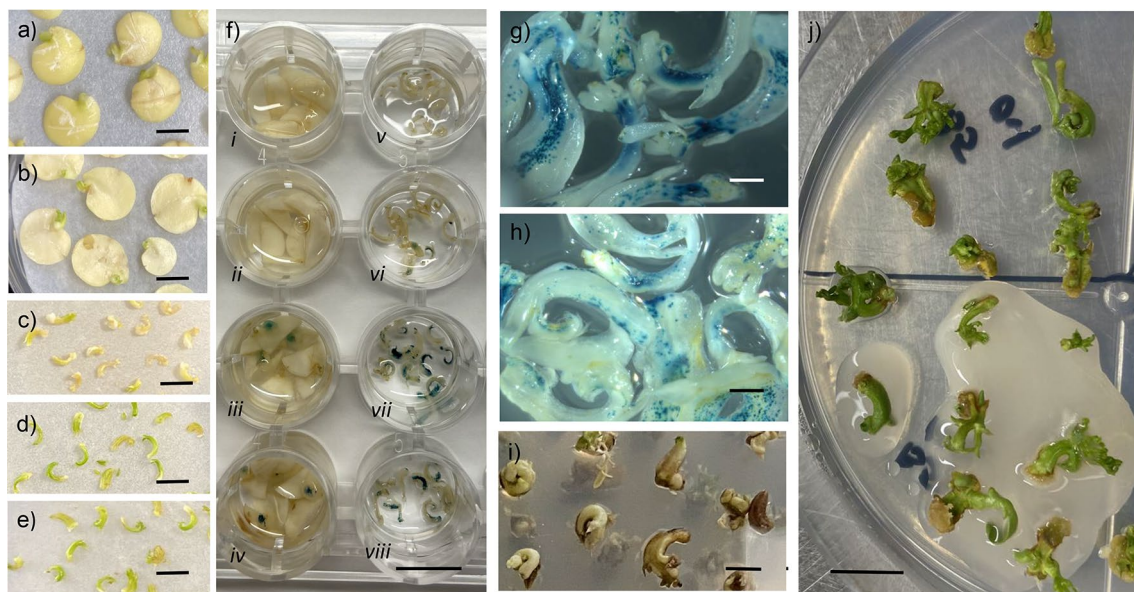


Fig. 1 **A, B** lentil cotyledons with wounded cotyledonary nodes attached at the end of 3 days of co-cultivation with **A** abaxial side up; **B** abaxial side down; **C–E** longitudinal slices of lentil embryo axes at the end of co-cultivation **C** on B5h medium; **D** on MS medium; **E** on B5 medium; **F** Explants after X-Gluc histochemical staining to show transient activity of the *gus* gene; left column (cotyledons with nodes) i, ii as from **A**; iii, iv as from **B**; right column (right column) v, as from **C**; vi as from **D**; vii, viii as from **E**; **G** histochemical *gus* stain-

ing of longitudinal slices of lentil embryo axes after 3 days of cultivation on B5 medium; **H** as in **G**, but after 4 days of co-cultivation; **I** explants on shoot induction medium that had been washed to remove excessive *Agrobacterium* contamination; **J** lentil explants on shoot induction medium explants from 3 day *Agrobacterium* co-cultivation (upper) and from 4 day co-cultivation (lower). Bars- **A–E I** = 5 mm; **F J** = × 10 mm; **G, H** = 500 μm

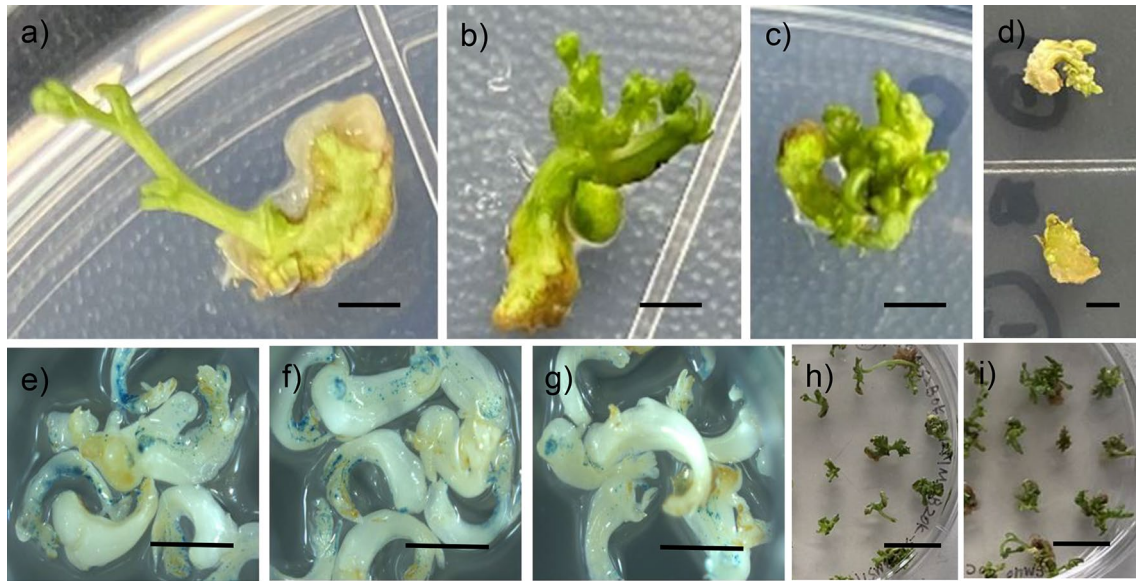


Fig. 2 **A** Lentil explant after co-cultivation on B5 co-cultivation medium, and 3 week exposure to 1 mg/l BAP for shoot initiation; **B** Explant as in **A** with a 3 week exposure to 2 mg/l BAP for shoot initiation; **C** explant as in **A** with a 3 week exposure to 2 mg/l BAP for shoot initiation; **D** Lentil explant after co-cultivation on B5h co-cultivation medium, and 3 week exposure to 2 mg/l BAP for shoot ini-

tiation and elongation; **E–G** Lentil explants histochemically stained for *gus* gene activity at the end of co-cultivation after sonication for **E** 2 s; **F** 5 s; **G** 10 s; **H** Control explants after 1 week in shoot development medium; **I** as in **H**, after exposure to 10 μ M lipoic acid during both the co-cultivation and the shoot initiation phases; Bars- **A–D**=2 mm; **E–G**=3 mm; **H I**=10 mm

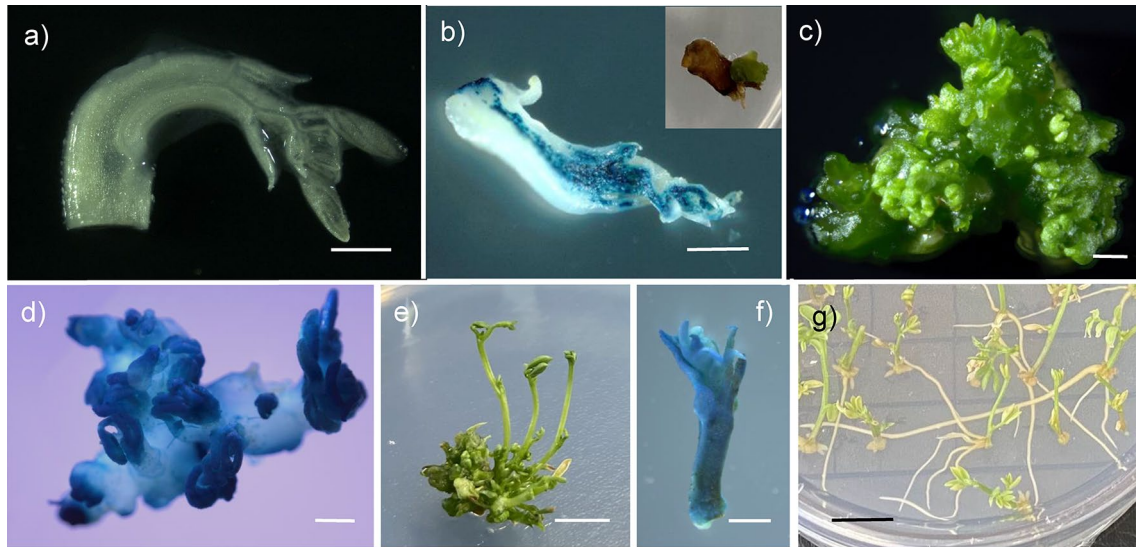
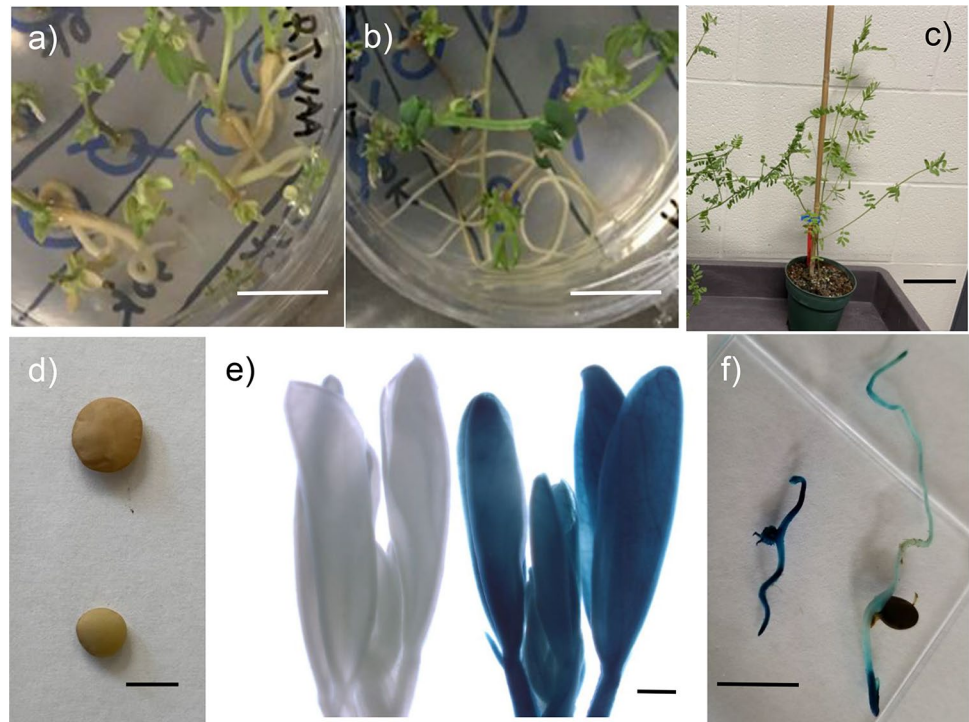


Fig. 3 Stages of tissue culture for transgenic lentil plant regeneration, based on best conditions tested; **A** An explant, a longitudinal slice through the shoot portion of the embryo axis of an imbibed seed, at the time of co-cultivation, with shoot apical meristem to the right. Bar, 500 μ m **B** An explant at the end of 3 days of co-cultivation after histochemical staining with X-Gluc to indicate expression of the *gus* reporter gene. Bar, 1 mm; insert is initial callus observed after 3 weeks on shoot elongation (SEM) medium **C** Cluster of shoots on

an explant after cultivation on SEM for 6 weeks. Bar, 1 mm **D** Histochemically stained callus with shoot cluster to indicated *gus* expression. Bar, 1 mm **E** Shoot cluster on shoot elongation media with three of the shoots at stage for placement on rooting medium. Bar, 1 cm **F** Histochemically-stained piece of shoot removed from a cluster similar to that in **E**. Bar, 1 mm. **G** Rooted shoots on rooting medium containing NAA (0.2 mg/l). Bar, 1 cm

Fig. 4 Rooted shoots, showing the difference in root morphology between those developing on medium with **A** NAA (0.2 mg/l) or **B** IBA (0.2 mg/l). Bar, 1 cm **C** Putative transgenic shoots after planting in soil. Bar, 10 cm **D** Lentil seeds from cv. Laird (above) and cv. Milestone (below) for size comparison. Bar, 5 mm **E** Histochemically X-Gluc stained leaflets from T₁ seedling, from a negative plant (left) and a plant positive for the inherited *gus* gene. Bar, 200 μ m **F** Histochemical X-Gluc stained T₁ seedlings to show inheritance of the reporter gene. Bar, 1 cm



In preliminary testing of lipoic acid, the explants exposed to lipoic acid at the lower concentrations of 5 and 10 μ M were similar to the controls, while those exposed to 50 μ M were paler green and smaller in size (data not shown). Due to a small explant number, histochemical staining for *gus* gene activity was not completed.

Agrobacterium concentration

To determine the best density of *Agrobacterium* suspensions to use, survival and transient expression with *gus* was balanced with the prevention of *Agrobacterium* overgrowth. The use of sterile filter paper on the surface of the co-cultivation medium to absorb excess suspension helped to ameliorate the latter. Suspensions with an optical density (OD) A_{660} of 0.1 and 0.2 were tested. At the higher OD, bacterial overgrowth was pervasive and effectively eliminated the explants of 90% of the individual plates. Washing with Timentin™ at the end of co-cultivation was explored; while the *Agrobacterium* was removed, the treated embryos grew much slower than embryos that had not been overgrown or treated (Fig. 1i). Contamination could also appear during shoot initiation and shoot elongation phases at this and at later stages (Fig. 1j) despite repeated transfers to fresh medium containing Timentin™.

The method of *Agrobacterium* application was also investigated. Using a suspension with an OD of 0.1, these

methods included soaking the explants for 20 min with gentle shaking or adding a 5 μ l droplet of the suspension individually to each explant. Both the soaking and the droplet methods resulted in similar transient expression of the *gus* gene. However, the former approach was less labour intensive and the latter more often resulted in *Agrobacterium* overgrowth.

Shoot initiation and development

Shoot initiation on explants co-cultivated on B5 medium was apparent with exposure to all three tested concentrations of BAP (1, 2 and 3 mg/l). After exposure to 1 mg/l BAP, individual shoots later developed and elongated (Fig. 2a). Using 2 mg/l BAP, a small cluster of shoots developed and start to elongate (Fig. 2b). With 3 mg/l BAP, small, tightly clustered shoots were observed (Fig. 2c). In contrast, explants co-cultivated on B5h media produced a mass of friable callus that did not differentiate (Fig. 2d), while explants on MS co-cultivation medium rapidly produced elongated, but non-transgenic shoots.

An incubation period of three weeks on the shoot initiation medium was determined to be suitable. While shoots were not yet visible at the end of this period, there appeared to be a residual carry-over of the influence of higher concentrations of BAP (2 and 3 mg/l) into subsequent growth. Use of lipoic acid in the shoot initiation medium, in combination

with the same concentration in the co-cultivation medium appeared to enhance shoot regeneration, with more shoots on a compact base (Fig. 2 d, e). Individual transgenic rooted shoots were obtained when 5 μ M or 10 μ M lipoic acid was included in both the co-cultivation and shoot initiation media. Unfortunately, these were small trials which were interrupted with a loss of material not caused by the treatments.

After 1 cycle on the shoot development medium which contained a reduced BAP concentration of 1 mg/l, small rounded calli were observed at the distal end of a small percentage (5–10%) of explants (Fig. 3b, insert). This smooth callus developed clusters of small shoots. The underlying surface callus which continued to enlarge eventually produced additional shoots (Fig. 3c). These masses were continually divided to encourage emergence of elongated shoots and the subdivisions from one explant could occupy more than 100 square centimeters if all material was maintained; however, all clusters were cut into pieces approximately 0.5 cm squared and only 5 to 6 were maintained per independent event. While the clusters did not uniformly exhibit *gus* staining, the developing and expanding areas on the shoots were distinctly blue (Fig. 3d). Individual shoots elongated within the clusters (Fig. 3e) and staining reveals continued *gus* activity (Fig. 3f). New shoots continued to develop and elongated from 4 until at least 7 months after the initial co-cultivation. Remaining material was discarded after 7 months in vitro or earlier if several rooted shoots had been successfully obtained.

During this phase, the kanamycin concentrations were gradually increased in 10 mg/l increments from 30 to 60 mg/l and then maintained at 60 mg/l through several rounds of selection. Occasionally, an explant would produce one de novo shoot at the apex of the explant. New axillary buds could expand at the distal end, which could out and survive on medium with 60 mg/l of kanamycin for 2 months before succumbing.

The production of multiple shoots on individual explants provided sufficient material for testing a variety of rooting conditions. Rooting of putative transgenic shoots (Fig. 3 g)

Table 3 Summary of the number of independent transgenic events regenerated per 100 seeds with *Lens culinaris* cv. Laird, using conditions outlined in Table 2

Experiment #	# of independent transgenic events
1	11
2	10
3	6
4	5
5	3
6	6
Average	6.8

was successful using freshly prepared IBA (0.2 mg/l), NAA (0.2 g/l) and a combination of both at the same concentrations. Over a series of 4 experiments using multiple shoots from putative transgenic calli, the rooting success was 70, 57 and 59%, respectively, for the three treatments (IBA, NAA, IBA+NAA). More than 90% of the shoot-producing calli yielding rooted shoots after 42 days on rooting medium. Not all the shoots from any one explant rooted. When tested, shoots that failed to root despite multiple subcultures on rooting medium did stain positive for *gus* activity along their length.

There were distinct differences noted in the morphology of the roots, with those developing after exposure to NAA (Fig. 4a) appearing abnormally thickened relative to the thinner roots, with more lateral branches that were obtained with IBA (Fig. 4 b). The roots those arising from the combination of two regulators closely resembled those with NAA alone.

When the shoots were at least 2.5 cm in length, they were transferred to soil (Sunshine 4) in 5 inch (12.7 cm) pots and continued growth under controlled conditions (Fig. 4c). They flowered and set seed which was collected for inheritance testing.

Using the identified parameters including sliced explants from the embryo axis, OD of 0.1 at 600 λ , media (Table 2) and timing as described above, 6 experiments with

Table 2 Summary of media identified for further testing of lentil transformation

	Co-cultivation	Shoot initiation	Shoot development/ elongation	Rooting
Basal medium	B5	MS	MS	B5 (half-strength)
Vitamins	B5	B5	B5	B5 (half-strength)
Growth regulators	–	BAP (2 mg/l)	BAP (1 mg/l)	IBA (0.2 mg/l)
Additives	Acetosyringone (100 μ M)	–	–	–
Kanamycin	0	25 mg/l	30 increasing to 60 mg/l	0
Sucrose	3%	3%	3%	3%
pH	5.8	5.8	5.8	5.8
Timentin	0	200 mg/l	200 mg/l	200 mg/l

Table 4 Summary of copy number for transgenic lentil lines, as determined from three different methods (n/d denotes not determined)

Plant ID #	ddPCR *	Southern **	qPCR **
Laird	0	0	0
5	1	0	1
6	1	1	1
7	1	1 (0–1)	1
14	3	n/d	2
22	2	2	4
23	2	2	1
24	1	1	1
25	7	> 3	4
28	1	n/d	1
29	3	n/d	2
30	3	3 (0–3)	1
33	6	3	10 (8–11)
34	1	3	1
36	2	1 (0–1)	4 (3–5)
37	10	3	14

*See Fig. 5 for visual representation and confidence interval

**Range of results between replicates in parentheses, where replicates differ

approximately 100 seeds per experiment were completed to validate the protocol. The average number of independent transgenic events identified was highly variable, often as influenced by aggressive overgrowth of *Agrobacterium*. Overall, the average frequency of success, based on the number of seeds sliced, was 6.8% (Table 3). This number does not include the multiple clones obtained from each explant. In addition, preliminary experiments were completed using seed from the cv. CDC Milestone. Seeds of cv. CDC Milestone were significantly smaller in size than that from cv. Laird (Fig. 4d) and it was more difficult to excise the explants; therefore, for this cultivar, the embryo axes were split in half and placed with the cut surface facing up on the medium. Four transgenic events were identified from four tests with 100 seeds; further experimentation would be required to determine a consistent frequency of success.

Analysis

In addition to tracking the reporter *gus* expression through histochemical staining, confirmation of incorporation of the inserted DNA and copy number were confirmed through molecular analyses. PCR was used to confirm the presence of selected section of the sequence from the *nptII* gene prior to planting and further analysis. Using DNA from PCR positive plants, relative to an untransformed control, three different approaches (ddPCR, non-radioactive Southern hybridization, and qPCR) were taken to resolve the best method for

copy number determination with this material. The results are summarized in Table 4. The ddPCR results from 15 independent events and a cv. Laird control (Fig. 5) show the copy number relative to the reference sequence set to 2 copies due to its homozygous nature. The confidence intervals indicate the consistency between statistical replications represented by the thousands of individual droplets. A sample of results obtained through the Southern hybridization show the poor clarity of the bands with poor contrast due to background signal (Fig. 6). The copy number determined by this approach was not consistent between the replicates, even using DNA from the same extraction and background; the ranges indicated in parentheses in Table 4. The qPCR results were highly variable between replicates. It should also be noted that only 2 replicates of the qPCR were completed as the advantages of ddPCR were quickly identified. The three methods were closest in agreement at the lower copy numbers with greater discrepancies with higher copy numbers.

Inheritance

The T₁ seeds (20 per line) of 10 independent events was planted for analysis to confirm inheritance, and to identify possible chimaeras. The number of plants per line varied based on germination rates. The testing was via *gus* histochemical staining of the young shoot material or entire seedling (Fig. 4 e ,f) and/or PCR analysis. The latter was used in two lines where no *gus* activity was observed in the progeny even though the T₀ parent plant was positive; PCR results for these two lines indicated that the sequence was inherited but not expressing. The results (Table 5) indicated that the number of PCR/*gus* positive plants from the single copy lines (identified by an *) regularly exceeded the expected 3:1 (positive:negative) ratio for inheritance of a single dominant gene.

Discussion

The intent of this study was to develop a reliable protocol for *Agrobacterium*-mediated transformation of *L. culinaris* (lentil). In the establishment of a protocol for the genetic transformation of any species, there is an endless list of variables that can be explored, including explant type, the DNA delivery system, the construct and *Agrobacterium* strain, types and level of chemical selection, basal media, and additives at each step. For the purposes of this study, information garnered from previous work in the lab (Polowick et al. 2000, 2004, 2014) was incorporated during initial steps; these included the use of acetosyringone in the cocultivation medium, and the use of a construct and *Agrobacterium* strain previously used successfully (LBG 66) with

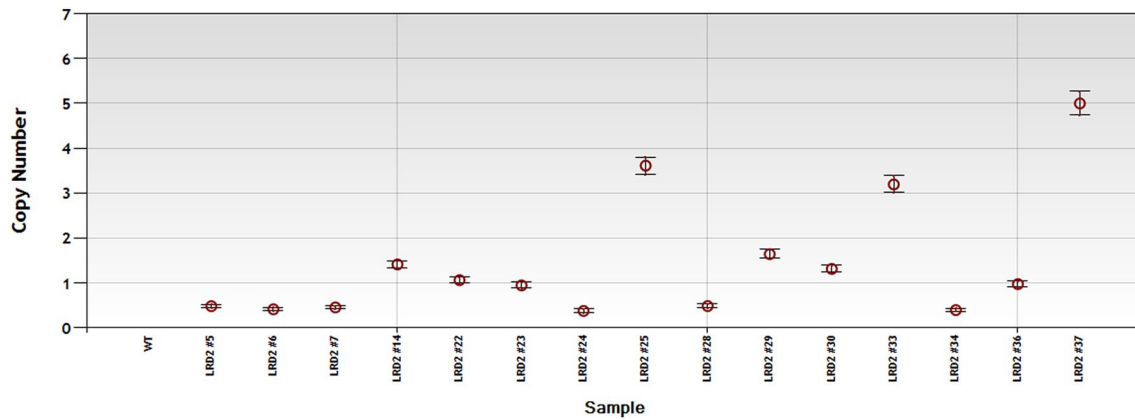


Fig. 5 Detection and copy number quantification of the inserted *gus* gene in 15 T0 lines of lentil using ddPCR showing the ratio of a probe from the construct to the homozygous, single copy unique reference sequence from cv. Laird; the reference sequence copy num-

ber was set to 2 to reflect its homozygous state. The Laird control is untransformed. The error bars represent the maximum and minimum Poisson distribution for the 95% confidence interval, as generated by the QuantaSoft™ software

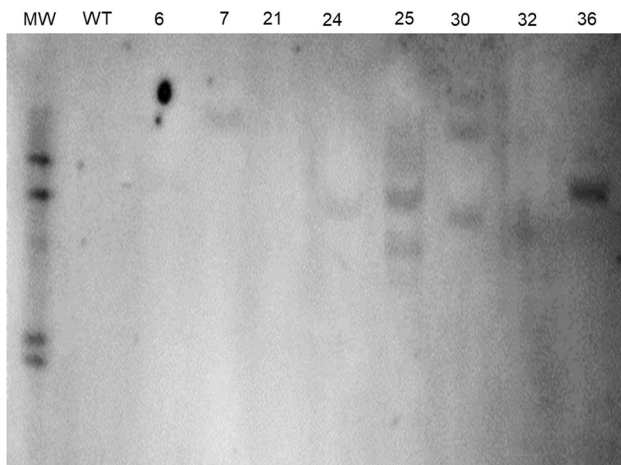


Fig. 6 Example of Southern hybridization results using DIG-labelled DNA on a selection of putative transgenic plants relative to the untransformed wild type (WT) and a molecular weight marker (MW)

kanamycin selection. Previous experience also guided some of the decisions on the starting points for media, selection and explant type.

Determining a suitable optical density of the *Agrobacterium* suspension and the duration of exposure to the culture requires consideration of the explant size, and should be sufficient for successful exposure without overgrowth and destruction of the explant. The continued rapid multiplication of the bacterium must also be reflected. The conditions in previous reports was variable, ranging from exposure to an OD of 0.8–1.0 for 20 min (Das et al. 2019) reduced from 45 min in their earlier work (Das et al. 2012) to an OD of 2.4 under vacuum for 20 or 30 min (Mahmoudian et al. 2002), although no transgenic plants were recovered from the latter. In this study, an OD of 0.1, with 20 min of incubation prior to placement on co-cultivation medium was found to provide the better transient *gus* expression, survival and healthy

Table 5 Inheritance of the transgene to the T1 generation

Line ID #	No of plants tested	GUS and/or PCR positive	Gus and/or PCR negative	Expected positive	ChiSqD*
6 *	17	14	3	12.75	0.1225
7 *	14	13	1	10.5	0.5952
13	15	15	0	n/a	n/a
22	11	7	4	n/a	n/a
23 *	13	10	3	9.75	0.0064
24 *	18	15	3	13.5	0.1667
25	11	10	1	n/a	n/a
28 *	16	13	3	12	0.0833
29	16	13	3	n/a	n/a
33	8	7	1	n/a	n/a
36	16	12	4	n/a	n/a

Chi-square analysis on the single copy lines (*) is based on an expected 3:1 Mendelian inheritance of a heterozygous, single copy, dominant gene (n/a not applicable)

explants, compared to a higher OD, or longer exposure. As experiments with the lowest number of transgenic events per 100 seed coincided with those in which *Agrobacterium* overgrowth had an impact, there still could be room for a reduction of the OD for further testing.

Sonication to induce micro-wounding small fissures in explant material and vacuum infiltration have been used to enhance the uptake of *Agrobacterium* tissues, especially into meristematic cells buried within explant tissues (Trick and Finer 1997). Chopra et al. (2012) incorporated 60 s of sonication and 5 min of vacuum infiltration into studies on lentil transformation using whole seed explants. While transient expression of the *gus* gene was reported in 68% of explants, the frequency of successful transformation was limited to 0.66%. Previous work in the lab (Polowick 2000, 2004, 2014) indicated that the wounding which occurred during slicing sufficiently exposed tissue for *Agrobacterium* uptake; combined with the presence of acetosyringone, this wounding appeared sufficient for DNA transfer. In the present study, the use of sonication reduced the transient expression of the *gus* gene, as visualized at the end of co-cultivation. The reduction was correlated with the duration of the sonication while the resulting wounds opened the explants to more extensive overgrowth of *Agrobacterium*.

Lipoic acid has been shown to enhance plant transformation efficiency, reduce the number of escapes that regenerate under selection and significantly reduce browning of tissues (Dan et al. 2009) which are problems with a number of legume species, although browning was not identified as an issue with lentil. In the current study, the addition of lower concentrations of lipoic acid to the co-cultivation and shoot initiation media in a preliminary test gave promising results; rooted shoots were recovered using both 5 and 10 μM in the co-cultivation medium. Both the transient expression of the *gus* gene and shoot initiation appeared to be improved. Unfortunately, limited resources prevented a thorough testing of a range of concentrations through the two separate stages.

The use of BAP for the production of multiple shoots *in vitro* is common. The concentration needs to be regulated, as high concentrations can later hamper rooting (Roberts and Schum 2003; Podwyszynska 2003). In varying concentrations, BAP has also been used for previous lentil transformation research (Gulati et al. 2002; Akcay et al. 2009). In contrast, but similar to the observations with micropropagation, Chopra et al. (2012) noted that, in comparison to TDZ, the presence of BAP in the medium in which the shoots were raised appeared to be inhibitory relative to later rooting success. In the current study, a 3 week exposure to BAP at a concentration of 2 mg/l was sufficient to induce clusters of multiple shoots which appeared at the next stage. Exposure to 3 mg/l BAP at this stage resulted in tight clusters of shoots that may have also developed

further; however, based on the anticipation that this may hinder rooting at a later date, the decision was made to continue with 2 mg/l BAP, as production of multiple shoots was consistent. The reduction to 1 mg/l BAP for subsequent regeneration allowed for the elongation of individual shoots in the cluster while still initiating further shoots as the cluster was separated into smaller pieces. Rooting did not appear to be severely limited by this level of BAP exposure. Although not every putatively transgenic shoot rooted, a number from each cluster showing *gus* gene activity were successfully rooted.

Rooting of lentil *in vitro* has been reported as a bottleneck in regeneration and transformation studies, as it is with other large seeded legumes. Some researchers have resorted to continued growth *in vitro*, including flowering (Das et al. 2019) but this resulted in very low seed set. Others (Gulati et al. 2002; Akcay et al. 2009) grafted the shoots onto lentil root stocks. This is a recurring issue with many large-seeded legumes. Chopra et al. (2012) achieved rooting on half strength MS (full strength vitamins) with 2.5 μM IBA (Phytigel) and selection at 20 mg/l kanamycin. Our previous experience with Phytigel and kanamycin selection in pea was that the effect of kanamycin was neutralized, yielding innumerable escapes (Polowick, pers. comm). This could explain some of the escapes observed with 0/6 or 4/14 of rooted shoots testing positive (Chopra et al. 2012).

In past studies in our laboratory, one of the defining features was rooting of both pea and chickpea in the presence of an increased concentration of selective agent, including kanamycin, L-phosphinothricin and hygromycin over the course of regeneration (Polowick et al. 2000, 2004). The absence of escapes was attributed to this continued selection. With tarwi, an Andean species of *Lupinus*, however, it was determined to be preferable to have the highest concentration of kanamycin (50 mg/l) during the shoot induction phase, with no selective pressure in the rooting medium; this kanamycin pressure at the earlier stage also eliminated escapes (Polowick et al. 2014). This practise may also have allowed for the survival, rooting and identification of a more transgenic shoots. In the current study, it was most effective to have the highest concentration of selection chemical (60 mg/l) for several 2 week rounds at the shoot development and elongation stage. Roots were obtained on half-strength MS basal medium and B5 vitamins, with NAA (0.2 mg/L) and IBA (0.2 mg/L), either individually or in combination. The morphology of the roots differed, with a thick, ropey appearance in the presence of NAA (Fig. 2a); therefore, it was not used for further experiments. Roots could require several cycles on rooting medium and some shoots later identified as transgenic, based on *gus* expression, failed to root. However, multiple rooted shoots were obtained for over 90% of the explants producing a transgenic, regenerative callus. This ability to produce multiple rooted shoots per event

may be advantageous for future testing for gene editing, as each shoot provides an opportunity for successful editing events; this is important in crop species where transformation frequencies are low.

Previous frequencies of success reported for lentil were, as with legumes in general, quite low and variable. Gulati et al. (2002) reported the highest range, from 3.1–6%, but this may include multiple shoots from one independent event as well as all plants transplanted to soil, several of which were later determined to be escapes. Other studies ranged from 0.66% (Chopra et al. 2012) using whole seeds, sonication and vacuum infiltration to 2.3% (Akçay et al. 2009). One group reported different results depending upon selection chemical: 0.35% with L-phosphthricin selection (Das et al. 2019) or 1.009% with kanamycin (Das et al. 2012). In the present study, we report an average of independent events as 6.8%, ranging from 3 to 11 events per experiment of 100 seeds.

Genotype dependence has been identified as an issue in genetic transformation of many crop species, including the legumes (Choudury and Rajam 2021). In addition, legumes are often referred to as recalcitrant in culture, a feature that has also been identified as genotype dependent (Ochatt et al. 2018). However, it is important not to equate regeneration capabilities to the ability to transform a genotype, as recognized by Iser et al. (1999) with wheat cultivars. Previous work from the lab on peas and chickpeas (Polowick et al. 2000, 2004) suggested that while there was variation in frequency, the established protocols were genotype independent. In the current study, only two cultivars were tested. Both cultivars proved transformable; limitations on the cv. CDC Milestone may have been largely to the smaller size of the seed, the ability to isolate the explant and a greater susceptibility to *Agrobacterium* overgrowth, also due to the small size. As the test cultivars were selected at random based on availability of seed, there is no reason to believe that the method would not work with other cultivars. Extension to other classes of lentil, such as small seeded red varieties remains to be tested, as much for the dextrous capability to manipulate the tissue as to determination of ability to transform and regenerate.

The copy number from the regenerated lines was investigated through several methods; Southern hybridization (DIG-labelled), qPCR and ddPCR. The ddPCR approach was determined to be the most consistent of the methods, especially in the case of higher copy number. It also proved less labour intensive. Similar comparisons reported in sugarcane by Sun and Joyce (2017) who pointed out that technical replicates are not required for ddPCR, as the sample is partitioned into thousands of individual droplets for micro-reactions. Each droplet is subjected to the ddPCR and, as such, is considered a statistical replicates. In contrast, several issues with Southern hybridization can cause problems with

resolution (Sun and Joyce, 2017), a problem experienced in the present study with the use of non-radioactive methodology. The ddPCR method has proven effective with several crops other species, including *Brassica* (Demeke and Eng, 2018), maize (Collier et al. 2017) and soybean (Iwobi et al. 2016), as cited by Giraldo et al. (2019), often in the context of testing feed and food for transgenic material.

In the present study, 40% of the transgenic lines were determined to be single copy insertions (Table 4; Fig. 3). Single copies are generally desired and selected for further line advancement. This is especially true for gene editing, where removal of the transgenes containing the gene editing machinery through segregation in subsequent generations is required. This is one of the advantages of *Agrobacterium*-mediated transformation relative to biolistic methods that more often result in multiple copies of the transgene in an event (Dai et al. 2001).

Few of the previous studies of lentil transformation reported inheritance into the T₁ generation or beyond. Akçay et al. (2009) confirmed stable transmission to the T₃ generation using PCR analysis and *gus* gene expression. Gulati et al. (2002) based inheritance of transgenes on leaf painting with herbicide and determined that none of the T₁ lines produced approached the expected number of transgenic offspring, based on Mendelian ratios. This is consistent with their expectation of chimaeras, possibly due to delayed introduction of the selection chemical. In the present study, stable transmission was observed into the T₁ generation and none of the lines produced fewer than the expected 3:1 ratio expected for inheritance of a dominant the expected 3:1 ratio of gene. For one line (#13), 15 of 15 seedlings were *gus* positive, potentially indicating a homozygous condition. Two lines were negative with respect to *gus* expression; however, the PCR still indicated the presence of the *nptIII* gene which suggested a loss of function in the reporter gene. Chi squared analysis was not completed for lines with multiple copies of the inserted gene, which could have been inherited individually or linked in tandem, thus skewing the expected numbers of positive plants in the T₁ generation.

Summary

The protocol for lentil reported here yielded rooted shoots from an average of 6.8 independent events per hundred seeds; this is above average for large seeded legumes. It also provided multiple shoots per event that could be advantageous in the production of gene edited plants. The expression of the reporter gene *gus* was visualized through histochemical staining, and further molecular analysis through PCR, qPCR, ddPCR and Southern hybridization confirmed transformation and provided copy number. Of the independent lines tested, 40% were single copy. Chimaeras were not

identified, as single inserted genes were inherited into the T₁ generation according to expected Mendelian ratios. At the same time, the protocol is labour intensive, especially in terms of explant excision. It took between 4 to 7 months from co-cultivation to the planting of rooted shoots, especially as the transgenic clusters continue to put out additional clonal shoots. While only two genotypes were tested, there is no reason to believe that some level of success would be limited to these randomly-chosen cultivars of green seeded legumes. The major limitation may only come in terms of the seed size and use with small-seeded red lentils remains untested. There is always room for improvement in protocols in order to increase the production of independent events. These can include changes in the promoters, the use of more potent *Agrobacterium* strains and/or the addition of other enhancers to the construct or medium and a reduction in the density of the *Agrobacterium* suspension, as well as expanded testing of genotypes.

Acknowledgements This project was supported by the Aquatic and Crop Resource and Development Centre as part of its contribution to the Sustainable Protein Production program of the National Research Council of Canada. The authors acknowledge the assistance of Daiqing Huang with assistance in identifying a unique reference sequence for the ddPCR.

Author contributions PLP: perceived, planned and oversaw and advised on the experiments, wrote the manuscript, prepared the Figures; WY: performed the experiments, provided input into approach, summarized the results, checked the manuscript, and participated in revisions. Both authors read and approved the final manuscript.

Funding Open Access funding provided by National Research Council Canada. This project was supported by the Aquatic and Crop Resource and Development Centre as part of its contribution to the Sustainable Protein Production program of the National Research Council of Canada.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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