



Establishment of *Agrobacterium tumefaciens*-mediated transformation of *Onobrychis viciifolia* Scop.

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Received: 9 March 2026 / Accepted: 11 May 2026 / Published online: 16 June 2026
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Abstract

Sainfoin (*Onobrychis viciifolia*) is a forage legume crop with resurgent popularity due to its anti-bloating and anthelmintic properties attributed to its relatively high content of condensed tannins. Although this property of sainfoin is desirable, sainfoin lags behind other popular forage crops in attributes, such as biomass yield, waterlogging tolerance, and stand persistence. Traditional breeding efforts to improve sainfoin have been constrained by its tetraploid genome and obligatory outcrossing nature. To accelerate the development of competitive cultivars using molecular-based approaches, a method for stable genetic transformation was needed. Here we describe a method for *Agrobacterium tumefaciens*-mediated transformation of sainfoin ‘AAC Mountainview’ cotyledons using *A. tumefaciens* strain GV3101 that produces an average transformation efficiency of 10%. We used the FB_35S::RUBY binary vector for transformation which harbors the *RUBY* reporter gene to enable quick and easy identification of transformation events early in the tissue culturing process. This research establishes the foundation for future advances in sainfoin through molecular-based improvement.

Key message

An *Agrobacterium tumefaciens*-mediated transformation method for the sainfoin ‘AAC Mountainview’ cultivar was established using the RUBY reporter to facilitate easy identification of transformation events.

Keywords Plant biotechnology · Transgenic plants · *Agrobacterium* · Legume · Sainfoin · Transformation method

Abbreviations

MES	2-morpholinoethanesulfonic acid
2,4-D	2,4-dichlorophenoxyacetic acid
MS	Murashige and Skoog
PVP	Polyvinylpyrrolidone
WT	Wild type

Introduction

Sainfoin (*Onobrychis viciifolia*) is a perennial forage legume crop with resurgent popularity over the last decade due to its anti-bloating and anthelmintic properties, which can be at least partially attributed to its relatively high proanthocyanidin content (Bhattarai et al. 2016; Legendre et al. 2017). Sainfoin is grown in pasture alongside the most widely grown forage, alfalfa (*Medicago sativa* L.), to prevent bloating in ruminants as a natural alternative to anti-foaming agents such as poloxalene (Foote et al. 1968; Wang et al. 2006). It has a nutrient profile similar to that of alfalfa; however, it has lower stand persistence, dry matter yield, and waterlogging tolerance making it costly and difficult to grow in many regions (Carlton et al. 1968; Sottie et al. 2017). Some success in improving sainfoin’s agronomic traits has been achieved through the development of improved cultivars such as ‘AAC Mountainview’, however these efforts have been constrained due to the crop’s obligatory outcrossing nature (Acharya 2015; Poudel et al. 2023). To overcome

Communicated by Heidi Halbwirth

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this, molecular-based strategies are needed to accelerate the development of competitive cultivars, but deployment of these strategies typically requires the establishment of a stable genetic transformation method. Although transformation of sainfoin has been achieved through the use of *Agrobacterium rhizogenes*, plants produced by this method had extensive morphological variations, limiting its use in studies (Golds et al. 1991). *Agrobacterium tumefaciens* has been used to genetically transform plants since the late 20th century, showing success in a wide variety of dicotyledonous species. However, there have been no reports in the literature on the use of this method for the stable transformation of sainfoin as of yet.

Reporter genes are often used to facilitate the development of transformation methods in plants as they allow for early monitoring of transgene expression; however, visualization of many of these reporters often requires expensive equipment or invasive techniques (He et al. 2020). Since leguminous species are commonly recalcitrant to transformation with minimal regenerants being recovered following transformation, the use of invasive visualization techniques hinders success in these species by destroying viable plant tissue (Nguyen et al. 2016; Nivya and Shah 2023). The *RUBY* cassette (He et al. 2020) is a reporter composed of three genes, *CYTOCHROME P450 76AD1* (*CYP76AD1*), *L-DOPA 4,5-DIOXYGENASE* (*DODA*), and *GLUCOSYLTRANSFERASE* (*GT*), that are separated by self-cleaving 2A peptides in a single reading frame. These three enzymes are required for the biosynthetic pathway that converts tyrosine to the pigment betalain, conferring a bright red colouration in transformed tissue offering a non-invasive and convenient approach for monitoring transformation events (He et al. 2020).

In this study, we established an efficient and reproducible *Agrobacterium tumefaciens*-mediated transformation method for the sainfoin ‘AAC Mountainview’ cultivar by adapting and optimizing protocols to overcome this species’ recalcitrance to transformation.

Materials and methods

Plant material and preparation

Seeds from the sainfoin ‘AAC Mountainview’ cultivar were obtained from Agriculture and Agri-Food Canada’s Research and Development Centre in Lethbridge, Alberta. To prepare plant material, seeds were dehulled and surface sterilized with 95% ethanol (vol/vol) for 1 min before being washed with 2% sodium hypochlorite (vol/vol) for 30 min at room temperature. Seeds were rinsed three times with sterile distilled water and plated on Murashige and Skoog (MS) medium (30 g/L sucrose, 8 g/L agar [PhytoTech], 4.33 g/L MS basal salts [PhytoTech], 1 mL/L 1000x MS vitamins [PhytoTech], pH 5.6) at 26 °C for three days in the dark to germinate. Germinated seeds were inserted into Magenta GA7 vessels (Merck KGaA, Darmstadt, Germany) filled with 100 mL of MS medium and placed in a growth chamber (21 ± 5 °C, 16 h photoperiod, 70% humidity, and light level of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$) for continued growth.

RUBY vector and bacterial strains

The FB_35S::RUBY vector was constructed by replacing the region between the left and right T-DNA borders in the pANICA6A vector (Mann et al. 2012) with a cassette containing a partially duplicated CaMV35S promoter, along with the *RUBY* coding region, and *Arabidopsis thaliana* heat shock protein 18.2 and *rbcS E9* transcriptional terminators from the 35S: RUBY vector (He et al. 2020) using standard cloning techniques. The resulting FB_35S::RUBY vector contains the *RUBY* reporter and the *NEOMYCIN PHOSPHOTRANSFERASE II* (*NPTII*) selectable marker gene which confers resistance to the antibiotic kanamycin within its T-DNA region (Fig. 1). The FB_35S::RUBY vector was introduced into the three *A. tumefaciens* strains GV3101, EHA105, and LBA4404 by the electroporation method as described by Nagel et al. (1990).

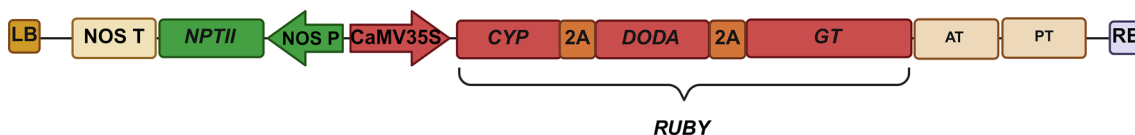


Fig. 1 Schematic of the FB_35S::RUBY T-DNA region. T-DNA elements from left to right: LB, left border sequence; NOS T, nopaline synthase terminator; *NPTII*, *NEOMYCIN PHOSPHOTRANSFERASE II* gene; NOS P, nopaline synthase promoter; CaMV35S, partially duplicated cauliflower mosaic virus 35S promoter; *CYP*, *CYTOCHROME P450 76AD1* gene; 2A, self-cleaving 2A peptide sequence;

DODA, *L-DOPA 4,5-DIOXYGENASE* gene; *GT*, *GLUCOSYLTRANSFERASE* gene; A.T, *Arabidopsis thaliana* heat shock protein 18.2 terminator; P.T; pea *rbcS E9* terminator; RB, right border sequence. This figure was created in BioRender. Pitambar, T. (2026) <https://BioRender.com/12158mw>

Explant preparation and pre-culture

Fifty cotyledon explants were prepared from 10-day old plants two days before infection. Explants were prepared by excising cotyledons from the plant and removing ¼ of the proximal and distal ends. Explants were cultured on MS0 media (30 g/L sucrose, 8 g/L agar, 4.33 g/L MS basal salts, 1 g/L polyvinylpyrrolidone [PVP], 1 mL/L 1000x MS vitamins, 100 mg/L ascorbic acid, 2 mg/L 2,4-dichlorophenoxyacetic acid [2,4-D], 0.25 mg/L kinetin, pH 5.6) and placed in the dark at 4 °C for 24 h. After 24 h, explants were plated on fresh MS0 media and placed in the dark at 21 ± 5 °C for 24 h.

Preparation of the *A. tumefaciens* culture

To prepare the *A. tumefaciens* culture for explant inoculation, a single colony was used to inoculate 3 mL of liquid LBS (10 g/L tryptone, 5 g/L yeast extract, 5 g/L sodium chloride, 50 mg/L kanamycin, 25 mg/L rifampicin, pH = 7.2) and incubated at 28 °C while shaking at 220 rpm for 24 h. A total of 200 µL of this culture was used to inoculate 100 mL of liquid LBS and incubated at 28 °C while shaking at 220 rpm for 24 h. Cultures were centrifuged at 3500 rpm at 20 °C for 15 min to pellet cells. The supernatant was discarded and cells were resuspended in AGI (10 g/L sucrose, 2.16 g/L MS basal salts, 1 mL/L 1000x MS vitamins, 500 mg/L 2-morpholinoethanesulfonic acid [MES], 39.2 mg/L acetosyringone, 15 mg/L dithiothreitol, pH = 5.5). The OD₆₀₀ of the culture (in stationary phase; OD₆₀₀ of 1.54–1.89) was measured and diluted to an OD₆₀₀ of 0.6. The culture was then incubated at room temperature while shaking at 50 rpm for 2 h in the dark.

Inoculation and co-cultivation of explants with *A. tumefaciens*

Explants were inoculated by submerging them in the *A. tumefaciens* suspension while shaking at 50 rpm for 15 min. Explants were then blotted dry with sterile filter paper and cultured on MSC medium (30 g/L sucrose, 8 g/L agar, 4.33 g/L MS basal salts, 1 g/L PVP, 1 mL/L 1000x MS vitamins, 500 mg/L MES, 39.2 mg/L acetosyringone, 15 mg/L dithiothreitol, 2 mg/L 2,4-D, 0.25 mg/L kinetin, pH = 5.5) for five days in the dark at 21 ± 5 °C. *A. tumefaciens* were then washed from explants with sterile Milli-Q water + 300 mg/L timentin three times before being blotted dry.

Regeneration of putative transformants

Following co-cultivation, explants were plated on MS1 medium (30 g/L sucrose, 8 g/L agar, 4.33 g/L MS basal

salts, 1 g/L PVP, 1 mL/L 1000x MS vitamins, 300 mg/L timentin, 100 mg/L ascorbic acid, 25 mg/L kanamycin, 2 mg/L 2,4-D, 0.25 mg/L kinetin, pH 5.6) to induce callogenesis. After one-month, red callus tissue was selected and subcultured separately on MS1 to remove competition from non-transformed tissue. After one-month, bright red calli were divided into pieces 0.5 cm in diameter and cultured on MS2 (30 g/L sucrose, 8 g/L agar, 4.33 g/L MS basal salts, 1 g/L PVP, 1 mL/L 1000x MS vitamins, 300 mg/L timentin, 100 mg/L ascorbic acid, 2 mg/L 6-benzylaminopurine, 0.1 mg/L 1-naphthaleneacetic acid, pH 5.6) to induce shoot organogenesis. When shoot initials formed, calli with shoots were divided taking care not to disrupt emerging shoots and cultured on MS2 in Phytatray II (Sigma-Aldrich) culture vessels in order to provide shoots room to grow. When shoots reached approximately 4 cm in length, they were excised from the callus and transferred to Magenta GA7 vessels filled with 50 mL of MS3 (10 g/L sucrose, 8 g/L agar, 2.15 g/L MS basal salts, 1 mL/L 1000x MS vitamins, 300 mg/L timentin, 0.02 mg/L 2-isopentyladenine, 0.02 mg/L indole-3-acetic acid, pH of 5.6) to root. Once roots developed to approximately 6 cm in length, they were removed from the medium and potted in a 1:1 mixture of vermiculite and soil. Pots were covered with a plastic bag and placed in a growth chamber (21 ± 5 °C, 16 h photoperiod, 70% humidity, and light level of 300 µmol m⁻² s⁻¹) for two weeks to acclimate. After two weeks, bags were removed and plants were allowed to grow to maturity.

Molecular characterization of putative transformants

Genomic DNA was extracted from putative transformants using the DNeasy Plant Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. A 948 bp fragment corresponding to the *RUBY* region of the FB_35S::RUBY vector was amplified by PCR using the forward primer 5'AACAGCATCCTTGAGTCTCTTCG3' and reverse primer 5'AAGCTTGTTTGGGGATCTAGTG3'. Reactions were setup using the following volumes: 1 µL genomic DNA (10 ng/µL), 3 µL of Milli-Q water, 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, 5 µL of Phire Direct PCR Master Mix (Thermo Fisher Scientific) to a total volume of 10 µL. PCR was conducted using the Eppendorf Mastercycler PRO S 6325 Thermal Cycler (Eppendorf, Hamburg, Germany) with thermal cycling conditions of 98 °C for 8 min, 45 cycles at (98 °C for 20 s, 58 °C for 10 s, 72 °C for 25 s), and a final extension at 72 °C for 90 s. PCR product was run on a 1% agarose gel containing 100 µL/L GelRed (Millipore Sigma) and visualized on a Gel Doc XR+ system (Bio-Rad, Hercules, United States). The remainder of the PCR product was purified using the

QIAquick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Purified PCR products were sent for Sanger sequencing at the London Regional Genomics Centre (London, Ontario, Canada). To verify sequence identity, sequencing data was aligned to the FB_35S::RUBY vector sequence using the MegAlign Pro software available at <https://www.dnastar.com/software/lasergene/megalign-pro/>.

Evaluating betalain production in sainfoin transformants

Relative betalain content in transgenic sainfoin was measured using a spectrophotometric method previously described by Pramanik et al. (2024). For this, 100 mg of leaf tissue was collected in PowerBead tubes (QIAGEN), and frozen with liquid nitrogen before being ground with the TissueLyser II (QIAGEN, Hilden, Germany). One millilitre of 50% methanol (vol/vol) was added to each PowerBead tube, tubes were vortexed for 30 s, then the solution was pipetted into a 2 mL microcentrifuge tube. The samples were centrifugated at 21,300 g for 5 min, then the supernatant was pipetted into a new 2 mL microcentrifuge tube. Samples were diluted using 50% methanol (vol/vol) until the solution's colour turned light pink to bring the sample's betalain concentration within the linear range of the standard. One hundred microlitres of each sample was pipetted in triplicate into 96 well plates, then absorbance was measured at 538 nm using a HTX Multimode Microplate Reader (Agilent, Santa Clara, Unites States). The relative betalain content in sainfoin transformants was calculated using the following equation:

$$\text{Betalain content} \left(\frac{\text{mg}}{\text{kg fresh tissue}} \right) = \left[\frac{A * DF * MW * 1000}{\epsilon * L} \right]$$

Where: A is the absorbance of the sample; DF is the dilution factor, calculated by dividing the volume of 50% methanol in μL used to dilute the ground tissue by the weight of the sample in mg; MW is the molecular weight of betalain, 550 g/mol; ϵ is the molar extinction coefficient of betalain in water, 60,000 L/mol*cm; L is the path length of the vessel used to measure absorbance.

Results

A. tumefaciens-mediated transformation of sainfoin

The establishment of a genetic transformation method for sainfoin using *A. tumefaciens* was carried out by exploring the capacity of three *A. tumefaciens* strains (GV3101,

LBA4404, and EHA105) to transform cotyledon tissue from the sainfoin 'AAC Mountainview' cultivar (Supplementary File 1). The *RUBY* reporter was employed to enable non-invasive identification of transformants during early stages of regeneration allowing for subculturing of primarily transformed tissue. Following the co-cultivation period, cotyledons maintained a healthy green appearance not exhibiting any signs of necrosis or browning (Fig. 2a). After one month of culture the number of calli expressing *RUBY* was counted to determine the number of stably transformed calli produced by each *A. tumefaciens* strain. *A. tumefaciens* GV3101 produced the highest number of calli expressing *RUBY* at 29.33%, whereas LBA4404 and EHA105 produced efficiencies of 13.33% and 5.33%, respectively (Table 1). Subculturing of *RUBY*-expressing callus tissue at one-month enabled recovery of primarily transformed calli after two-months of culture (Fig. 2b). Once transferred to MS2 medium, calli developed shoot initials within 2 weeks (Fig. 2c). However, shoot initials only developed in calli produced by cotyledons transformed with *A. tumefaciens* GV3101, while regeneration failed to progress beyond the callus stage in calli produced by cotyledons transformed with EHA105 or LBA4404. Multiple shoots developed in *RUBY*-expressing calli, exhibiting varying intensities and patterns of red pigmentation (Fig. 2d). An average of 1.08 shoots per callus developed normal leaves (Fig. 2e), whereas many of the other shoots formed abnormally developed leaves. Shoots approximately 4 cm in length with well developed leaves were transferred to MS3 medium for rooting. On this medium, shoots began to elongate and multiply while callus formed at the base. Root growth was initiated from this callus and took approximately one to two months to grow to at least 6 cm in length (Fig. 2f). Only 30% of shoots rooted, producing an average of five plants per 50 explants used giving a transformation efficiency of 10% (Table 1). The rate of survival for potted plants (Fig. 2g) was 100%, with no observed difficulties during acclimation to greenhouse conditions.

Molecular analysis of transgenic sainfoin

PCR was conducted to verify the presence of the *RUBY* transgene in putative transformants after acclimatizing to greenhouse conditions. Genomic DNA from one primary transformant per independent transformation trial *OvRC-2*, *OvRC-3*, and *OvRC-4* were used for PCR analysis, with the negative control using genomic DNA from wild type (WT) sainfoin, and the positive control using FB_35S::RUBY vector DNA. PCR was conducted twice per putative transformant to produce a high enough concentration of PCR product for Sanger sequencing. Each of the three plants produced bands at 948 bp, the same as the positive control

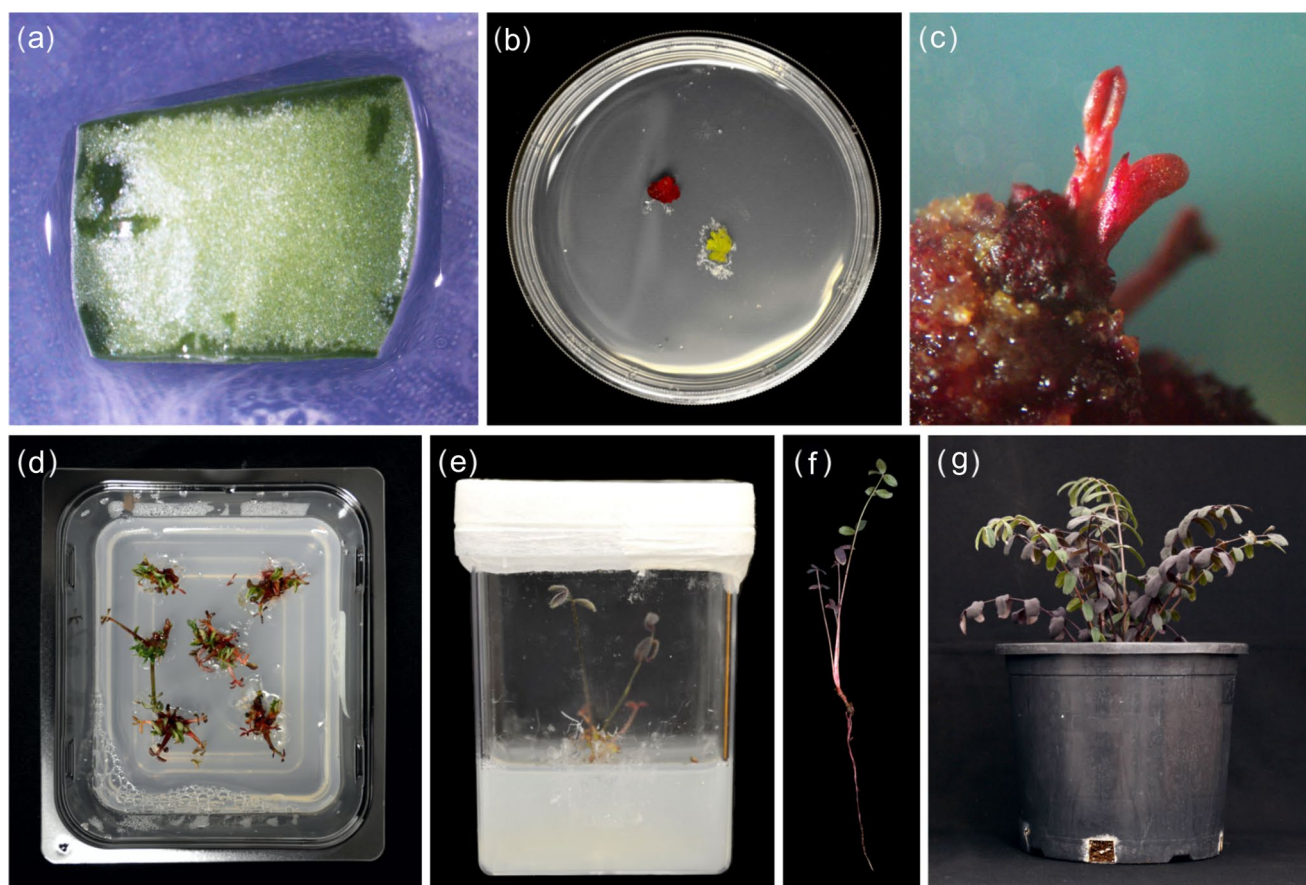


Fig. 2 Regeneration of putative sainfoin transformants expressing the *RUBY* reporter. **A)** Cotyledon explant one day after co-cultivation with *A. tumefaciens*; **B)** *RUBY*-expressing calli (red) and control calli (green) 2-months after co-cultivation with *A. tumefaciens*; **C)** Shoot initials emerging from callus tissue 3-months after co-cultivation with

A. tumefaciens; **D)** Shoot growth 4-months after co-cultivation with *A. tumefaciens*; **E)** Shoot growth 5-months after co-cultivation with *A. tumefaciens*; **F)** Rooted plantlet 6-months after co-cultivation with *A. tumefaciens*; **G)** Mature *RUBY*-positive sainfoin 7-months after co-cultivation with *A. tumefaciens*

Table 1 Transformation efficiencies of *Agrobacterium tumefaciens* strains in sainfoin

<i>A. tumefaciens</i> strain	Initial Explants	<i>RUBY</i> -expressing calli (%)	<i>RUBY</i> -positive plants	Transformation efficiency (%)
GV3101	50	29.33±2.18	5±0.47	10±0.94
LBA4404	50	13.33±1.09	0	0
EHA105	50	5.33±0.54	0	0

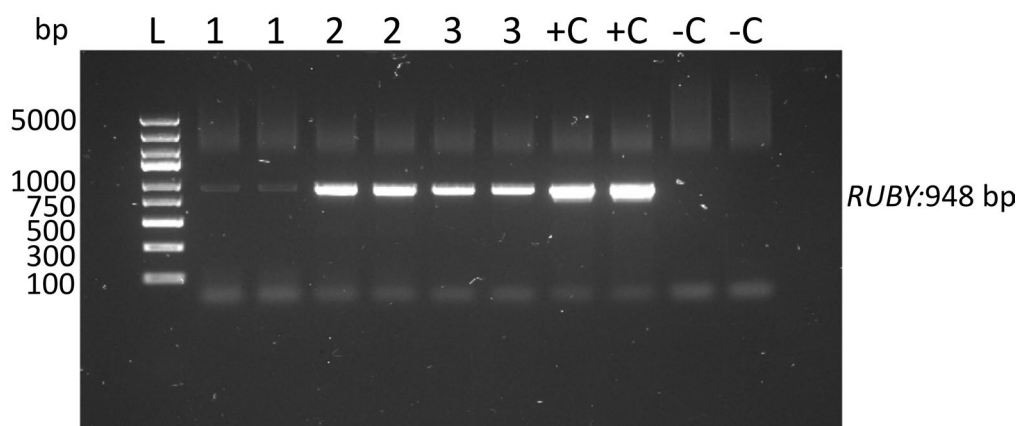
Data represent the mean±standard error of three independent experiments using 50 explants each

lane (+C), while the negative control produced no bands (Fig. 3a). Sanger sequencing of PCR amplicons was aligned to a 50 bp region of the *RUBY* reporter, which showed no variance in sequence identity (Fig. 3b).

Accumulation of betalain in transgenic sainfoin plants

To quantify the activity of *RUBY* enzymes in sainfoin transformants, relative betalain content in the leaves of WT and *RUBY*-positive plants was calculated using spectrophotometry. In mature plants expressing the *RUBY* reporter, leaves, stems, and roots showed a red tint, which was notably different than the bright green colouration observed in WT sainfoin (Fig. 4a, b). Spectrophotometric analysis indicated an absorbance of 0 at 538 nm for each of the WT sainfoin samples, whereas the sainfoin transformants had absorbances ranging from 0.115 to 0.250. An equation using the molar extinction coefficient of betalain was referenced to calculate the relative betalain content in each plant, which ranged from 119.65 to 260.12 mg/kg of fresh weight in the transformants (Fig. 4c).

(a)



(b)

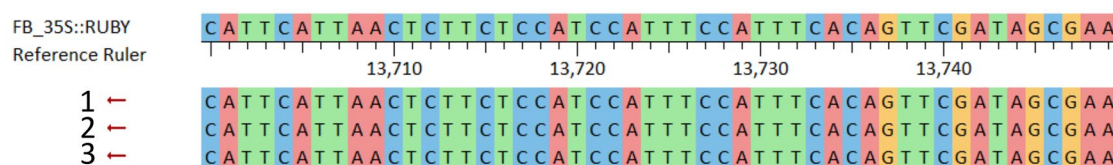


Fig. 3 Analysis of transgenic plants by PCR and Sanger sequencing. **A)** Electrophoresis of PCR amplicons on a 1% agarose gel. L: the SM1563 ladder (Thermo Scientific, Waltham, United States); 1: PCR amplicon generated using genomic DNA from *OvRC-2_1*; 2: PCR amplicon generated using genomic DNA from *OvRC-3_1*; 3: PCR

amplicon generated using genomic DNA from *OvRC-3_1*; +C: PCR amplicons generated using FB_35S::RUBY vector DNA as the positive control; C: PCR amplicons generated using WT sainfoin genomic DNA as the negative control; **B)** Sanger sequencing of PCR amplicons aligned to a 50 bp region of the *RUBY* reporter

Discussion

A. tumefaciens is a powerful tool for introducing genetic changes into plants; however, recalcitrance in some leguminous plants remains a hindrance to their improvement. Initial attempts at transforming the leguminous crops soybean (*Glycine max*) and chickpea (*Cicer arietinum*) showed transformation efficiencies of 6% and 0.4% respectively, which have since been increased to 10% and 17.56% respectively, through ongoing research efforts (Gupta et al. 2023; Hinchey et al. 1988; Krishnamurthy et al. 2000; Li et al. 2017). During this study we found that of the three *A. tumefaciens* strains used for transformation, GV3101 was the only one capable of producing stable transformants, having a transformation efficiency of 10%, comparable to that achieved in soybean. A notable difference between GV3101, LBA4404 and EHA105 is that GV3101 harbors the pMP90 helper plasmid, which contains the *trans-zeatin-synthesizing* (*tzs*) gene encoding trans-zeatin synthase, an enzyme that catalyzes the production of the cytokinin trans-zeatin (Han et al. 2013). Hwang et al. (2010) found that GV3101 with a loss of function mutation in the *tzs* gene produced

lower transformation efficiencies in Arabidopsis, and supplementing the media with cytokinins during co-cultivation improved the transformation efficiencies of *A. tumefaciens* strains without the *tzs* gene by two to five-fold. It is possible transformation of sainfoin using *A. tumefaciens* EHA105 and LBA4404 can lead to stable transformants if the co-cultivation media were supplemented with an appropriate level of cytokinin.

Previous reports showed that rooting of calli-derived shoots of sainfoin cultivars typically occurs at a frequency of 60%–80% (Garshasbi et al. 2012; Özcan et al. 1996; Turgut et al. 2022), but in the current study only 30% of the transgenic shoots developed roots. Cytokinins and auxins play a complex role in cell fate determination in plants, often through direct or indirect regulation of one another to tightly control activity during developmental stages (Alvilez-Montalvo et al. 2022; Kolachevskaya et al. 2021; Li et al. 2011). Cytokinins induce organogenesis by regulating the expression of genes involved in histone modification such as *CYTOKININ INDUCED ROOT WAVING 2*, which in turn upregulates the expression of auxin biosynthesis genes, some of which have been shown to be involved in root

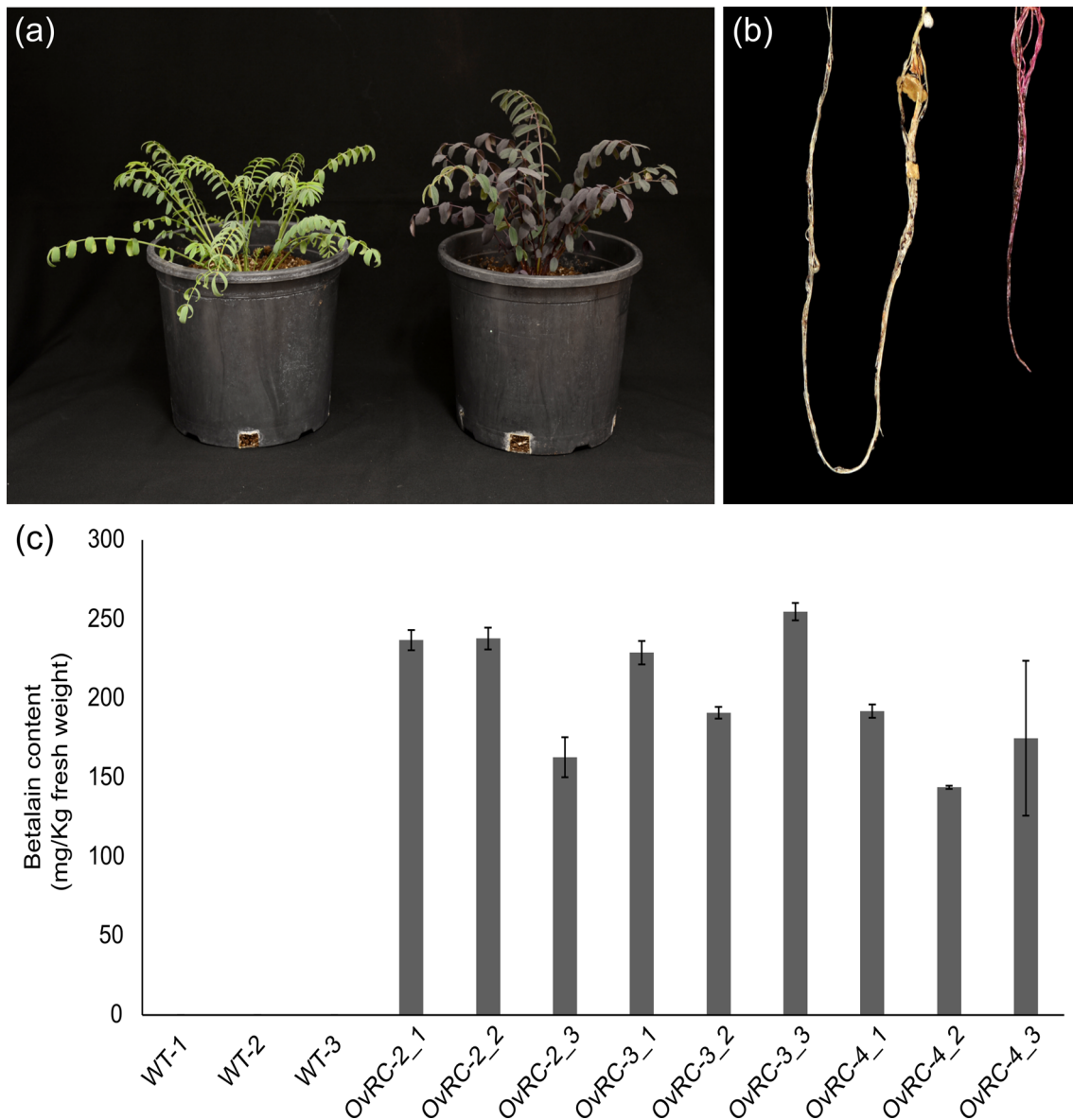


Fig. 4 Phenotypic and chemical comparison of WT and *RUBY*-positive sainfoin plants. **A)** Left: WT sainfoin with bright green colouration. Right: *RUBY*-positive sainfoin with reddish-purple colouration; **B)** Left: WT sainfoin roots with pale yellow colouration. Right: *RUBY*-positive sainfoin roots with pinkish-red colouration; **C)** Betalain content in WT and *RUBY*-positive sainfoin from three independent trans-

formation events (*OvRC2*, *OvRC3*, *OvRC4*). Betalain content in WT plants measured at 0 mg/kg of fresh weight, whereas betalain content in each of the nine transformants ranged from 119.65–260.12 mg/kg of fresh weight. Error bars represent the standard deviation between sample measurements

development in *Arabidopsis* (Jones et al. 2010; Zhang et al. 2012). During pathogenic infection, plant tissue increase the production of salicylic acid, leading to epigenetic changes in the genome, which have been shown to specifically dysregulate the expression of genes involved in root initiation in pearl millet (*Pennisetum glaucu*) (Ngom et al. 2017; Rai et al. 2018). In our study, sainfoin explants were exposed to both *A. tumefaciens* and cytokinins throughout the duration of transformation and regeneration, thus it is possible that this long period of exposure caused undesired pleiotropic

effects, including the inhibition of root growth. Future research on this interaction is needed to better understand how genome plasticity is impacted by exposure to *A. tumefaciens* to optimize this method and circumvent difficulties in regeneration.

RUBY was an effective reporter for developing this transformation method, as tissue began manifesting red colouration within a week of infection by *A. tumefaciens*. This allowed for early subculturing of only putative calli transformants, reducing competition for nutrients by untransformed

cells. Although not the primary goal of this study, betalains also present biological benefits to sainfoin and the animals that consume it. Betalains have been shown to be involved in plant stress response to high salinity with increased production of betalains corresponding to increased tolerance in *Disphyma australe* (Jain and Gould 2015). This could expand the territory in which sainfoin is grown as a forage crop, as intolerance to high salinity is one of the crop's notable weaknesses (Parlak and Parlak 2008). When cattle were fed *RUBY*-expressing alfalfa forage, enteric methane production in cattle was reduced (Heuschele et al. 2026). Future research should be directed towards studying the benefits that *RUBY*-expressing sainfoin could have when used as forage for cattle.

In conclusion, this study presents the first reported instance of stable genetic transformation of *O. viciifolia*, which was achieved through *A. tumefaciens*-mediated transformation. These findings set the foundation for future studies in functional gene characterization and crop improvement in this species.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11240-026-03486-z>.

Acknowledgements We would like to acknowledge Alex Molnar at the Agriculture and Agri-Food Canada London Research and Development Centre for his assistance with graphics. This study was funded by a grant from The Beef Cattle Research Council.

Author contributions TAP designed the study, conducted the experiments, analyzed the data and wrote the manuscript. SDS provided the genetic material and secured the funding. LC contributed to experimental design. SEK co-supervised the project and reviewed the manuscript. AH supervised the project and contributed to the experimental design and manuscript preparation.

Funding Open access funding provided by Agriculture & Agri-Food Canada library. This research was funded by The Beef Cattle Research Council through SCAP-ASC-01 Beef Cluster Activity 20 A - Advancing Canadian sainfoin breeding for profitability, animal health, and a future of sustainable beef and forage production systems.

Data availability The data supporting the findings of this study are available within the manuscript. Any additional datasets generated and analysed during the current study are available from the corresponding author upon request.

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

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