



Research article

A hairy-root transformation protocol for *Trigonella foenum-graecum* L. as a tool for metabolic engineering and specialised metabolite pathway elucidation

Constantine Garagounis^{a,*}, Konstantina Beritza^{a,1}, Maria-Eleni Georgopoulou^{a,1}, Prashant Sonawane^b, Kosmas Haralampidis^c, Alain Goossens^{d,e}, Asaph Aharoni^b, Kalliope K. Papadopoulou^a

^a Department of Biochemistry and Biotechnology, Laboratory of Plant and Environmental Biotechnology, University of Thessaly, Biopolis, 41500, Larissa, Greece

^b Faculty of Biochemistry, Department of Plant Sciences, Weizmann Institute of Science, 7610001, Rehovot, Israel

^c Faculty of Botany, Department of Biology, National and Kapodistrian University of Athens, 15701, Athens, Greece

^d Ghent University, Department of Plant Biotechnology and Bioinformatics, 9052, Ghent, Belgium

^e VIB-UGent Center for Plant Systems Biology, 9052, Ghent, Belgium

ARTICLE INFO

Keywords:

Fenugreek

Trigonella foenum-Graecum

Hairy root

Agrobacterium rhizogenes transformation

TSAR

Triterpene saponin activating regulator

ABSTRACT

The development of genetic transformation methods is critical for enabling the thorough characterization of an organism and is a key step in exploiting any species as a platform for synthetic biology and metabolic engineering approaches. In this work we describe the development of an *Agrobacterium rhizogenes*-mediated hairy root transformation protocol for the crop and medicinal legume fenugreek (*Trigonella foenum-graecum*). Fenugreek has a rich and diverse content in bioactive specialised metabolites, notably diosgenin, which is a common precursor for synthetic human hormone production. This makes fenugreek a prime target for identification and engineering of specific biosynthetic pathways for the production of triterpene and steroidal saponins, phenolics, and galactomanans. Through this transformation protocol, we identified a suitable promoter for robust transgene expression in fenugreek. Finally, we establish the proof of principle for the utility of the fenugreek system for metabolic engineering programs, by heterologous expression of known triterpene saponin biosynthesis regulators from the related legume *Medicago truncatula* in fenugreek hairy roots.

1. Introduction

Metabolic engineering of crop plants is rapidly expanding in response to increased demand for greater crop yields and for enhanced, affordable production of high-value specialised plant metabolites (Reed and Osbourn, 2018; Lau et al., 2014; Lu et al., 2016). This expansion is aided by the advent of new high-throughput cloning methods (Engler et al., 2014; Sarrion-Perdigones et al., 2011) and large-scale transcriptomic dataset acquisition, in conjunction with metabolic network modeling and prediction (Skraly et al., 2018). Nonetheless, in spite of great progress in these areas, and in contrast to unicellular chassis organisms in which metabolic engineering has made huge gains recently (Nikel and de Lorenzo, 2018; Antonovsky et al., 2016; Gleizer et al., 2019), many crop plants cannot yet be exploited in this manner, partly due to the lack of established transformation methods for many species.

Thus, if crop plants are to be fully exploited both for increased biomass yields and as a source and platform for production of desirable metabolites, the need to develop reproducible, straightforward transformation protocols for crop plants becomes an obvious priority.

Trigonella foenum-graecum (fenugreek) is an important leguminous crop and feedstock plant that is also widely used for pharmaceutical purposes traditionally and in the medical industry. Fenugreek is an annual legume of the Fabaceae family, indigenous to countries of the Mediterranean, Middle East, Northern Africa, and Asia (Altuntaş et al., 2005; Flammang et al., 2004). Seeds and leaves of the plant are widely used as forage, food and spice, but most importantly, for their medicinal properties (Flammang et al., 2004; Petropoulos, 2002), which include: anti-diabetic (Kan et al., 2017; Jaiswal et al., 2012; Gaddam et al., 2015; Knott et al., 2017), hypocholesterolemic (Gaddam et al., 2015; Knott et al., 2017), antioxidant (Khole et al., 2014; Kenny et al., 2013;

* Corresponding author.

E-mail address: kgkaragkounis@bio.uth.gr (C. Garagounis).

¹ These authors contributed equally.

Szabo et al., 2018; Belguith-Hadriche et al., 2013), anticancer, anti-metastatic (Chiang et al., 2007; Chen et al., 2011; Li et al., 2010; Srinivasan et al., 2009; Sethi et al., 2018), and anti-microbial (Sharma et al., 2017; Norziah et al., 2015) activity. Major metabolic constituents of fenugreek, which contribute to its pharmaceutical traits, include 95 different steroidal and triterpene saponins and their precursor aglycones (Kang et al., 2013), notably the sterol diosgenin. Fenugreek also contains a plethora of: phenolics, alkaloids, fibers—specifically high amounts of galactomanans—vitamins and minerals (Flammang et al., 2004; Khole et al., 2014; Norziah et al., 2015; Zandi et al., 2014; Acharya et al., 2008; Mehrafarin et al., 2010; Fedacko et al., 2016; Rababah et al., 2004; El Nasri and El Tinay, 2007; Raghuram et al., 1994; Wang et al., 2012; Jani et al., 2009). The nutritional and pharmacological importance of these metabolites makes fenugreek a prime target for crop trait improvement, plant metabolic engineering, and novel compound mining. However, in order to capitalize on these diverse biosynthetic pathways, it is necessary both to draw on the knowledge available from model legumes on specialised biosynthetic processes and to develop the tools necessary for the genetic manipulation of fenugreek.

To achieve the above goal, we developed an *Agrobacterium rhizogenes*-mediated hairy root transformation protocol for fenugreek. Hairy root cultures offer an alternative system enabling the large-scale and highly efficient production of desirable bioactive plant-derived compounds (Lu et al., 2016) that can be used in parallel with heterologous transformation platforms, such as tobacco leaf infiltration (Reed and Osbourn, 2018) or introduction of plant biosynthetic pathways into unicellular organisms (Kung et al., 2018; Arendt et al., 2017; Pyne et al., 2019). The hairy root phenotype in plants is caused by a Gram-negative soil bacterium, *Agrobacterium rhizogenes*, which infects plants causing hairy root disease (Riker et al., 1930) by inserting a T-DNA derived from the root-inducing (Ri) plasmid into the plant genome (Chilton et al., 1982) and, similar to *A. tumefaciens*, is able to transfer T-DNA from disarmed binary vectors into the plant genome in parallel with the Ri T-DNA. This co-transformation enables the production of transformed roots containing genes of interest as well as the Ri-derived T-DNA (Hooykaas et al., 1977; Sukhapinda et al., 1987). Contrary to *A. tumefaciens*-induced calli, *A. rhizogenes*-infected plants can undergo organogenesis, and form new roots transformed with a desired T-DNA without external phytohormone application as these are produced ectopically by genes encoded in the Ri T-DNA (Chilton et al., 1982; Estruch et al., 1991). It is thus an important, low cost, simple and efficient system for genetic engineering established in many, often recalcitrant to transform, plant species (Gomes et al., 2019; Meng et al., 2019), including legumes (Chen and Otten, 2017; Siefkes-Boer et al., 1995; Peraza-Luna et al., 2001; Boisson-Dernier et al., 2001; Merkli et al., 1997; Cho et al., 2000; Stiller et al., 1997; Aggarwal et al., 2018; Stougaard et al., 1987; Díaz et al., 1989; Lee et al., 1993; Diouf et al., 1995; Kereszt et al., 2007; Quandt, 1993; Diaz et al., 2000; Estrada-Navarrete et al., 2007; Sinharoy et al., 2009; Bonaldi et al., 2010; Clemow et al., 2011; Imanishi et al., 2011; Mellor et al., 2012; Spano et al., 1987). Some works suggest that hairy root transformation is feasible in fenugreek (Merkli et al., 1997; Shahabzadeh et al., 2014). However, the method has not been utilised in the relevant literature. Combined with the availability of fenugreek transcriptomic datasets and the identification of diosgenin biosynthesis genes (Christ et al., 2019), this transformation method could be used in identifying and engineering a multitude of specialised metabolite biosynthetic pathways.

We further adapted a promoter from the model legume *Lotus japonicus Ubiquitin1* gene (pLjUBQ1) (Maekawa et al., 2008) to drive transgene expression in this crop legume and validated both our transformation method and the functionality of this heterologous promoter in our new system. To do so, we expressed GFP and two triterpene saponin biosynthesis activating regulator (TSARs) transcription factors from *Medicago truncatula* (Mertens et al., 2016a) as a test case

for modulating the ratio of triterpenoid and steroidal saponins produced by fenugreek.

2. Materials and methods

2.1. Seed sterilization and germination

Fenugreek cultivar Co 1 and *Lotus japonicus* cultivar Gifu B129 seeds were scarified with sulfuric acid (H₂SO₄) for 7 min to aid imbibition (optionally, this step was omitted for fenugreek without a noticeable difference in germination), then washed 4 times with pre-chilled dH₂O and surface-sterilized with gentle shaking in a solution containing 20% (v/v) commercial bleach, 81 µM Tween-20 for 20 min. Sterile dH₂O was added and seeds were imbibed for 16 h under dark conditions in 4 °C. Seeds were placed onto 1% (w/v) agar in square petri dishes in a growth chamber (22°C) under dark conditions for 24 h. Plants were grown in a 16 h day/8 h dark cycle at 22–23 °C degrees.

2.2. Bacterial strains and plasmids used

E. coli DH5a was used for routine cloning and plasmid propagation. *E. coli* DB3.1 was used to propagate *ccdB* suicide gene-containing Gateway vectors. Two *A. rhizogenes* strains, ARqua1 and LBA9402, were used and were routinely grown in LB medium in the presence of appropriate antibiotics. The binary vector *p35S::eGFP*, corresponding to *pUB-GWS-GFP* (Maekawa et al., 2008), carrying a green fluorescent protein gene [eGFP S65T variant (Chiu et al., 1996; Niwa et al., 1999)] under the control of the CaMV35S promoter was introduced into both *A. rhizogenes* strains. The *pUBI::GFP::Hyg* vector was constructed by PCR-amplification of the GFP coding sequence from *pUB-GWS-GFP* in order to incorporate attB recombination sites at each end. This PCR product was used in sequential BP Gateway Clonase (Thermo Scientific) reaction with the *pDONR207* vector (Thermo Scientific) followed by LR reaction into the *pUB-GW-HYG* Gateway destination vector (Maekawa et al., 2008). *pUBI::TSAR1/2::Hyg* vectors were constructed by direct BP clonase reactions of the *pUB-GW-HYG* destination vector with the linearized expression vectors *TSAR1:pK7WG2D* and *TSAR2:pK7WG2D* (Mertens et al., 2016a). The *pX11* vector for betalain biosynthesis is described in Polturak et al. (2016).

2.3. Hairy root transformations

Lotus japonicus hairy root transformations were carried out as described in (Delis et al., 2011; Krokida et al., 2013) using the *pUBI::TSAR1/2::Hyg* and *pUBI::GW::Hyg* control plasmids transformed into *A. rhizogenes* LBA1334. Individual transgenic roots harboring the above constructs were identified by PCR on genomic DNA. The newly developed fenugreek hairy root transformation protocol is described below.

2.4. Induction of hairy roots in fenugreek

Seedlings at 4 days post germination were used for transformation. 500 µL of each *Agrobacterium* strain grown to saturation in liquid LB was spread on solid LB medium in Petri dishes containing appropriate antibiotics and incubated for 24 h at 28 °C to generate a dense bacterial slurry. The seedling radicles were cut with a scalpel (~3 mm from radicle tip) and the traumatized radicle surface was coated with the appropriate *Agrobacterium* strain by scraping some of the desired bacterial slurry. The seedlings were then placed in large petri dishes containing half strength MS medium [2.2 g/L Murashige & Skoog salts including vitamins (from Duchefa Biochimie, Belgium), 1% (w/v) sucrose, 0.05% (w/v) MES, 0.6% (w/v) agar, pH adjusted to 5.7 using KOH]. Up to 6 seedlings were placed on a single square (10 × 10 cm) dish. The dish was placed at an angle of approximately 45° for 3–4 days in a 20 °C growth chamber (16 h light/8 h dark). After four days, the seedlings were transferred to half strength MS medium plus 400 µg/mL ampicillin

to eliminate *Agrobacterium* growth. At this point antibiotic/herbicide selection can be added to ensure regeneration of uniformly transformed roots. However, initial experiments in which hygromycin was added to culture medium at concentrations starting from 12.5 µg/mL showed that the resistance gene (conferring hygromycin resistance) and driven by the 35S promoter in the T-DNA vectors we used (from Maekawa et al., 2008; Supporting Fig. S1) did not confer resistance to fenugreek callus and hairy roots (Supporting Fig. S5). Lacking a selection gene under control of a suitable promoter, no selection was added at this stage in subsequent experiments. This procedure was repeated once a week. The plants were kept in a 20 °C growth chamber until roots appeared (about 12–14 days after infection). Alternatively, MS-medium was substituted with McCown's Woody Plant medium supplemented with 3% (w/v) sucrose, 0.05% (w/v) MES (pH 5.7) with similar results. Healthy seedlings with regenerated roots were transferred to pots containing 2:1 sand:vermiculite and watered with half strength Hoagland's nutrient solution [full strength Hoagland's composition: MgSO₄ 2 mM, KH₂PO₄ 1 mM, FeEDTA 0.1 mM, KNO₃ 10 mM, Ca(NO₃)₂ 4H₂O 5 mM, H₃BO₃ 46 mM, MnCl₂ 9 mM, ZnCl₂ 0.8 mM, CuCl₂ 0.2 mM, H₂MoO₄ 0.1 mM].

Root regeneration and callus formation ability of the two *A. rhizogenes* strains, ARqua1 and LBA9402, were tested periodically. Genomic DNA from regenerated hairy roots was extracted using a CTAB solution [100 mM Tris-HCl pH 8, 20 mM EDTA pH 8, 1.4 M NaCl, 55 mM cetyltrimethyl ammonium bromide (CTAB), 1% (w/v) PVP], followed by phenol/chloroform extraction and ethanol precipitation. Positive transgenic hairy roots were verified by PCR, using primers targeting either the 35S promoter of the hygromycin resistance gene or the GFP CDS (Supporting Table S1). The reaction conditions were: Initial denaturation at 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min.

2.5. Microscopy imaging

Transformed roots expressing GFP were further identified by fluorescence detection using an MZ10F dissecting microscope from Leica (Solms, Germany) equipped with a mercury UV-lamp and an eGFP band-pass or a DsRed2 long-pass filter. Confocal Laser Scanning Microscopy (CLSM) was carried out on an Axiovert Observer microscope with an LSM800 confocal module from Zeiss (Jenna, Germany), using a 488 nm diode laser line for excitation and collecting an emission wavelength range of 515 ± 15 nm, with the pinhole set to 1.0 airy unit and detector settings set to collect the full dynamic range of emitted fluorescence.

2.6. Sequence and phylogenetic analysis

HMGR and MYB bHLH protein sequences were retrieved from TAIR (<https://www.arabidopsis.org/index.jsp>) for Arabidopsis, LotusBase (<https://lotus.au.dk>) for Lotus, and from the assembled transcript dataset described in (Christ et al., 2019) for fenugreek. Three HMGR full-length coding sequences were identified in the *Lotus* genome (both versions 2.0 and 3.0), amplified from *Lotus japonicus* cDNA and sequences compared to those predicted on Lotus Base (Supporting Table S2). For comparison, sequences were aligned using Clustal Omega (Sievers et al., 2011). The bHLH maximum likelihood phylogenetic tree was constructed on the phylogeny. fr server (<http://www.phylogeny.fr/index.cgi>) (Dereeper et al., 2008, 2010) using MUSCLE (Edgar, 2004) for alignment, GBLOCKS (Castresana, 2000) to remove poorly aligned sequence blocks, PhyML (Anisimova and Gascuel, 2006; Guindon and Gascuel, 2003) for tree construction, and TreeDyn (Chevenet et al., 2006) for visualization.

2.7. Measuring gene expression levels by qPCR in transformed hairy roots

Frozen regenerated fenugreek or *Lotus japonicus* roots, identified as

transformed by PCR, were ground under liquid nitrogen and used to extract total RNA using a modified LiCl procedure (Brusslan and Tobin, 1992). The expression levels of *TSAR1*, *TSAR2* (Mertens et al., 2016a) and predicted transcripts for fenugreek HMGR (Chaudhary et al., 2015) and *CAS* (Ciura et al., 2017) and putative *Lotus japonicus* HMGRs1-3 were estimated by Luna® Universal One-Step RT-qPCR (New England Biolabs), using specific primers for each gene. To normalize the expression results, the translation *Elongation Factor1α* (*EF1-α*) (Wang et al., 2012) was used as an endogenous gene previously identified in fenugreek RNA sequencing. *Lotus japonicus Ubiquitin1* was used as an internal reference for qPCRs on *Lotus japonicus* RNA as described in (Delis et al., 2011). Two to three technical replicate reactions were carried out per sample. The reaction conditions were: reverse transcription at 55 °C for 10 min and then 40 cycles of 95 °C for 10 s, 60 °C for 30 s and melt-curve at 65–95 °C with increment 0.5 °C every 5 s. Data analysis was performed according to the ΔC_T method (Livak and Schmittgen, 2001), with all data being expressed as mean ± SD (standard deviation).

2.8. Metabolite extraction and analysis

Root tissue (~100 mg per sample), derived from individual transformed roots (verified by PCR targeting T-DNAs) growing on composite hairy root plants ~6 weeks post *A. rhizogenes* infection, was ground to fine powder under liquid nitrogen using a mortar and pestle. 300 µL of extraction solvent (80% v/v MeOH, 0.1% v/v formic acid) was added to each sample and homogenized by vortex for 30 s, followed by sonication in a bath sonicator for 20 min and again vortex for 30 s. Insoluble material was removed by centrifugation 20,000 × g, 15 min at 4 °C and the supernatant transferred to new tubes. Prior to UHPLC-MS analysis, samples were filter sterilized using PTFE syringe filters with 0.22 µm pore size. Saponin content in these extracts was analysed as reported previously (Cardenas et al., 2016).

2.9. Statistics

All pairwise comparisons were done using Student's two-tailed homoscedastic *t*-test in Microsoft Excel™ using a significance threshold of 0.05.

3. Results

3.1. *A. rhizogenes* ARqua1 is suitable for fenugreek hairy root generation

In order to develop a reproducible protocol and identify suitable *Agrobacterium rhizogenes* strains for the transformation and the successful expression of transgenes in fenugreek, we chose to use the binary vector *p35S::eGFP*, containing GFP under the control of the commonly used constitutive CaMV35S promoter (Supporting Fig. 1). This vector was transformed into two different *A. rhizogenes* strains that have been successfully used to transform legumes in the past, *A. rhizogenes* ARqua1 (Boisson-Dernier et al., 2001) and LBA9402 (Stiller et al., 1997). Fenugreek seedlings were infected following the procedure outlined in the corresponding methods section (summarized in Fig. 1). Infection with both strains led to callus formation and root regeneration in each of two independent experiments, but with different success rates for each strain. Specifically, infected plants were monitored for the rate of callus formation and root regeneration from the day of infection until ~3 weeks (21 days) later and for transformation efficiency after 21 days post infection (dpi) (Table 1). Over the course of two experiments 43% and 57% of explants infected with the ARqua1 strain and 70–76% of those infected with LBA9402 formed callus after 12 dpi (Table 1 and Fig. 2A, arrows). The number of explants forming callus did not change greatly at least until 21dpi. Regenerated roots could be observed by naked eye at 14 dpi (Fig. 2A, arrowheads) and the number of explants with roots increased until 21

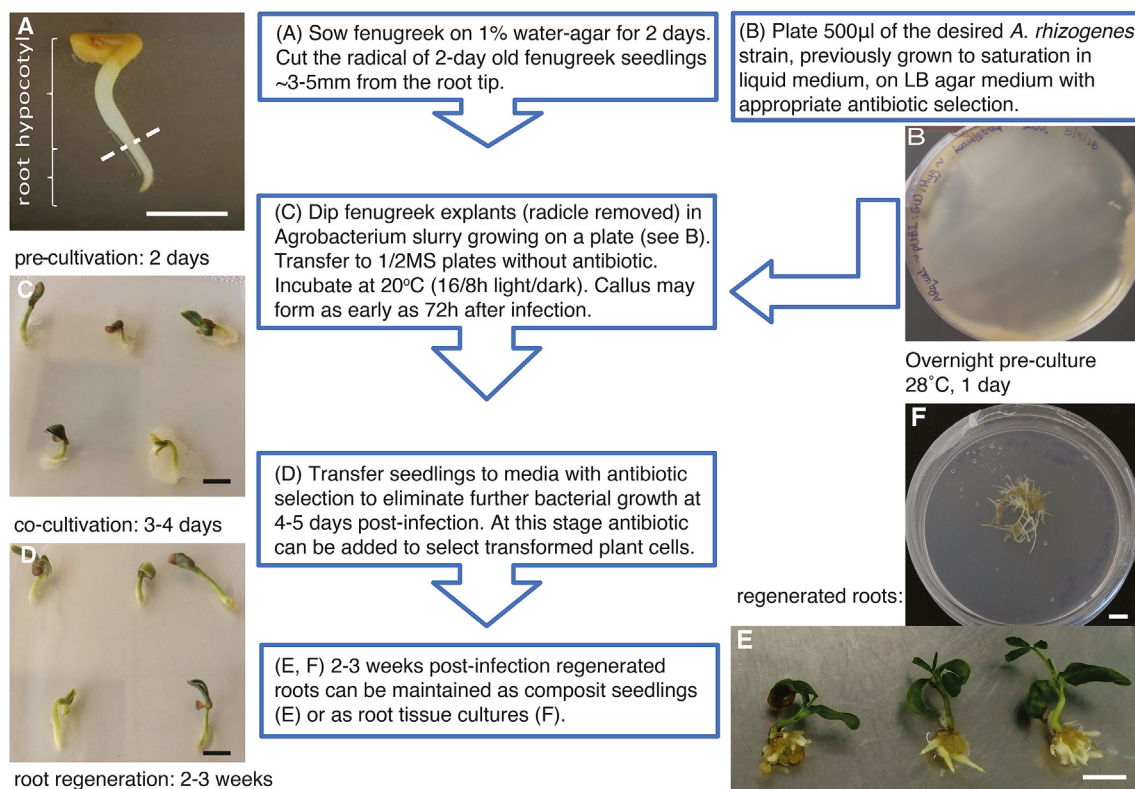


Fig. 1. Protocol flowchart describing the procedure for fenugreek hairy-root transformation. The dashed line indicates the recommended point for radicle excision. Scale bars in all images are 1 cm.

dpi. ARqua1 infected explants started with 10% and 25% root regeneration at day 12 and reached 30% and 47% by day 17 in the two experiments, respectively. LBA9402-infected explants correspondingly started from 0 to 5% on day 12 and reached 12% and 28% on day 17. Plants with regenerated hairy roots could either be grown as composite plants with an untransformed shoot and regenerated transformed root, or roots could be excised from explants and grown rapidly in tissue culture on solid (Fig. 2B and C) or in liquid medium (Fig. 2D). Roots grown on solid medium displayed growth rates of up to 1 cm/day (Fig. 2B, arrowheads), indicating that this method can be used to achieve rapid accumulation of hairy root biomass.

From 21 dpi onwards, regenerated roots were sampled and screened

for GFP expression on a dissecting microscope equipped for fluorescence detection. However, no clear GFP fluorescence was detectable in the regenerated roots using this method (Supporting Figs. S2A–C), suggesting that either regenerated roots were not transformed with the T-DNA, or that the *CaMV35S* promoter used to drive GFP expression is not fully functional in fenugreek. We therefore extracted genomic DNA from regenerated roots and screened them for T-DNA integration using a PCR to target a fragment of the GFP coding sequence (Supporting Figs. S2D and E). This approach showed that regenerated roots were indeed transformed with the *p35S:GFP* construct. The ARqua1 strain had a 27–43% and the LBA9402 strain a 7–28% transformation efficiency over the two experiments, thereby demonstrating that: (1) A.

Table 1

Results of fenugreek hairy root transformation using two different *A. rhizogenes* strains¹.

| ARqua1 ^a | Experiment 1 | | | Experiment 2 | | | |
|----------------------|--------------|------------------|-------------------|--|------------------|-------------------|--|
| | Days | Callus Formation | Root regeneration | Transformation efficiency ^b | Callus Formation | Root regeneration | Transformation efficiency ^b |
| ARqua1 ^a | D12 | 56.57 | 25 | 48.33 | – | 9.52 | 26.98 |
| | D15 | 56.57 | 41.67 | | 42.86 | 19.5 | |
| | D17 | 56.57 | 46.67 | | 42.86 | 30.16 | |
| | > D17 | 56.57 | 46.67 | | 42.86 | 33.33 | |
| LBA9402 ^a | Experiment 1 | | | Experiment 2 | | | |
| | Days | Callus Formation | Root regeneration | Transformation efficiency ^b | Callus Formation | Root regeneration | Transformation efficiency ^b |
| LBA9402 ^a | D12 | 73.33 | 5 | 28.33 | – | – | 6.67 |
| | D15 | 73.33 | 21.67 | | 70 | 6.67 | |
| | D17 | 73.33 | 28.33 | | 76.67 | 11.67 | |
| | > D17 | 73.33 | 33.33 | | 76.67 | 13.33 | |

– Indicates that no root or callus was visible at this time point. Some roots do emerge in the absence of visible callus and are often non-transformed.

^a ARqua1 and LBA9402 harboring the *p35S:eGFP* vector. Values shown are percentages of the total number of seedlings infected with each strain/construct. Sixty plants were used for each construct in each experimental replicate.

^b Transformed roots are defined as the regenerated hairy roots in which the presence of the T-DNA was confirmed by PCR.

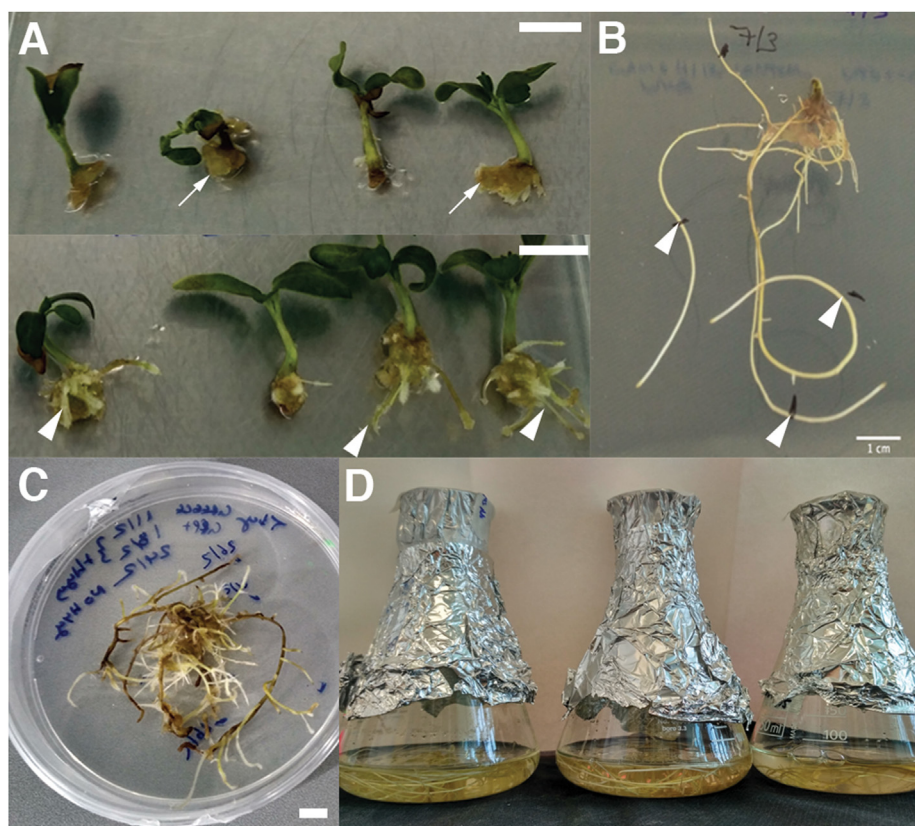


Fig. 2. Regeneration and culture of fenugreek hairy roots after infection with *A. rhizogenes*. A) Fenugreek explants ~14 days after infection with *A. rhizogenes* ARqua1. Arrows indicate callus formed around the infection site; arrowheads indicate emerging roots. B) Fenugreek hairy roots after shoot excision, grown on McCown's woody plant medium supplemented with sucrose as a carbon source for heterotrophic growth. The distance between each arrowhead and the corresponding root tip was covered in three days of growth. C) Representative images of fenugreek hairy roots grown in petri dishes on solid, and D) in flasks with liquid McCown's woody plant medium. Scale bars in all images are 1 cm long.

Table 2
Results of two independent fenugreek hairy-root transformation experiments.

| Experimental replicate | 1 | 2 | 1 | 2 |
|--|----------------------|-----------------------|-----------------------|-----------------------|
| <i>A. rhizogenes</i> strain ^a | ARqua1 (pLjUBQ:eGFP) | ARqua1 (empty vector) | ARqua1 (empty vector) | ARqua1 (empty vector) |
| Callus formation | 66.7 | 42.0 | 71.7 | 81.7 |
| Root regeneration | 30.0 | 43.3 | 45.0 | 58.3 |
| Transformation rate | 21.7 | 35.0 | 45.0 | 38.9 |
| Transformation rate (GFP) | 11.66 | 20.0 | – | – |
| Fluorescent roots ^b | 50.0 | 57.7 | – | – |

^a *Agrobacterium rhizogenes* ARqua1 transformed with pLjUBQ:GFP construct and an empty control vector were used to transform fenugreek hairy roots. Unless otherwise indicated, values shown are percentages of the total number of seedlings infected with each strain/construct. Sixty plants were used for each construct in each experimental replicate.

^b Percent of individual regenerated transformed hairy roots expressing GFP (transformation efficiency).

rhizogenes ARqua1 transforms fenugreek hairy roots more efficiently than LBA9402 with this approach, and (2) although this transformation method is successful, the *CaMV35S* promoter is not suitable to drive strong transgene expression in fenugreek roots.

As a further test of this latter hypothesis, we also transformed fenugreek hairy roots with the binary vector *pX11*, encoding three enzymes that are sufficient to engineer the heterologous production of red betalain pigments from beetroot (Polturak et al., 2016). Two of three genes for betalain production encoded by the *pX11* vector are controlled by *pCaMV35S*, while the third gene is controlled by the *Arabidopsis Ubiquitin 10* promoter (Polturak et al., 2016). Similar to our previous result with the *p35S::eGFP* construct, although the three genes could be amplified from *pX11*-transformed root DNA, production of betalain pigments was not detectable by eye (Supporting Fig. S3). This finding supports our hypothesis that the 35S promoter does not

function well in fenugreek.

3.2. The *Lotus japonicus Ubiquitin1* promoter is functional in fenugreek

Since successful transgene expression is critical in any type of metabolic engineering application, we set out to identify a promoter that is functional in fenugreek. Lacking a publicly available sequenced fenugreek genome, we set out to identify a suitable promoter from the literature. Researchers often utilize polyubiquitin-derived or other endogenous housekeeping gene promoters from the target or closely related plant species (Jang et al., 2002). Thus, we chose the *Lotus japonicus Ubiquitin1* promoter, which has been previously characterized and performs well in binary vectors used to transform *Lotus japonicus* (Maekawa et al., 2008).

We cloned the same eGFP sequence used previously under the control of the *LjUBQ1* promoter in a T-DNA also carrying a *pCaMV35S*-driven hygromycin selection cassette (*pUBQ:eGFP:Hyg*, Supporting Fig. S1). This vector was transformed into *A. rhizogenes* ARqua1 in parallel with an empty T-DNA control vector and used to generate fenugreek hairy roots in two independent experiments. Callus formation, root regeneration, and root transformation efficiency were roughly similar to the previous experiments with *p35S::eGFP* in ARqua1 (Table 2), although in both of these experiments the empty vector control had slightly higher efficiency for all of these parameters.

In order to identify the presence of the T-DNA in putative transformed roots, a PCR analysis of genomic DNA was again performed for all regenerated roots as before. Transformation efficiency (the percentage of regenerated roots that was transformed based on PCR screening) was $76.4 \pm 4.3\%$, with an overall transformation rate (the percentage of infected explants that led with transformed regenerated roots) reaching $28.3 \pm 6.6\%$. Conversely, the transformation efficiency based on eGFP fluorescence was $53.8 \pm 3.9\%$ of individual regenerated roots in the absence of hygromycin selection, while the overall transformation rate based on GFP expression rather than on PCR

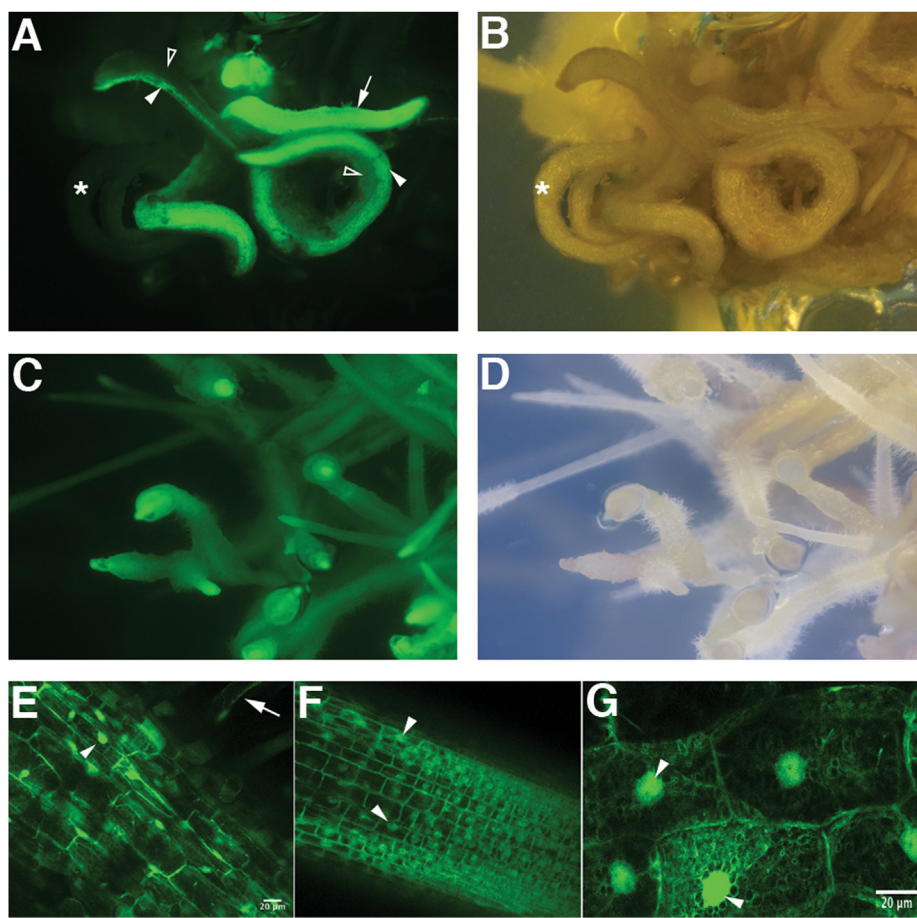


Fig. 3. GFP fluorescence in regenerated fenugreek hairy roots. A) GFP and B) bright field images of regenerated hairy roots grown without selection three weeks after infection with the *A. rhizogenes* ARqua1 pLjUB:GFP strain. Arrowheads and arrows in A indicate segments of chimeric regenerated hairy roots expression or not expressing GFP, respectively. The asterisk in A and B indicates a representative regenerated root with non-detectable GFP fluorescence. C) GFP and D) Bright field image of GFP-expressing hairy roots, cultured heterotrophically on McCown's woody plant medium, 7 weeks after infection with *A. rhizogenes*. E-G) CLSM fluorescence images of untagged GFP expressed in regenerated fenugreek hairy roots under the control of the *LjUBQ1* promoter. Nucleus-like structures (arrowheads) and root hair fluorescence (arrow) are indicated.

(i.e., the percentage of infected explants that led to regenerated GFP-expressing roots) was $15.8 \pm 5.9\%$, over both experiments (Table 2, Fig. 3A–D, and Supporting Fig. S4). Within transformed roots, strong GFP expression was detectable in all cell types, including: epidermal and root hair, cortical cell (Fig. 3E–G), vascular bundle and root tip/cap and detached epidermal cells (Supporting Fig. 3F–H). In individual cells, eGFP localized primarily to the cytosol and nucleus (Fig. 3E–G, arrowheads), as is expected for a heterologous soluble protein lacking an organelle targeting sequence. Nuclear localization is expected in this case, because proteins less than 40 kDa in size can readily diffuse into the nucleus (Timney et al., 2016). Taken together, these results indicate that the *LjUBQ1* promoter is indeed functional in fenugreek and useful for constitutive expression of transgenes in multiple cell types.

Although many of the regenerated roots uniformly express GFP (Fig. 3A, arrow), some roots do not fluoresce at all (Fig. 3A, asterisk), confirming that not all roots are transformed with the GFP construct. Crucially, some regenerated roots display chimeric expression of GFP, with one longitudinal half of the root expressing GFP (Fig. 3A, white arrowheads) while the other half does not (Fig. 3A, open arrowheads). In some extreme cases, only a few cells in a regenerated root display fluorescence (Supporting Fig. S4). This finding demonstrates that regenerated hairy roots do not arise from one specific cell, but multiple callus cells, and are therefore chimeric with regard to transgene expression or even transformation with the transgene, similar to previous studies using hairy roots (Limpens et al., 2004; Sinharoy et al., 2015). Even though the pLjUBQ1:eGFP construct constitutes a good marker in itself, we also wanted to utilize an antibiotic selection marker to facilitate the selection of uniformly transformed hairy roots. We tried to exploit the 35S driven hygromycin resistance gene present in our T-DNA construct by growing transformed hairy roots on a medium containing hygromycin. We found that roots stopped growing within one

week and eventually died (Supporting Fig. S5), once again confirming that the 35S promoter is not functional in fenugreek. It is therefore critical for appropriate selectable markers under the control of the *LjUBQ1* or similarly functional promoters to be developed for use in this transformation system.

3.3. Heterologous expression of TSARs in fenugreek hairy roots influences the expression of endogenous genes

Given that the ultimate goal of this work was to develop a standard transformation procedure for metabolic engineering applications in fenugreek, we wanted to validate our method by using known regulators of legume specialised metabolism. Overexpression of TSAR1 and TSAR2 increases the accumulation of non-hemolytic and the hemolytic triterpene saponins, respectively, while suppressing expression of cycloartenol synthase (CAS), the phytosterol and steroidal saponin biosynthesis gene, in *Medicago truncatula* (Mertens et al., 2016a). Thus, we hypothesized that overexpression of *MtTSAR1* and *MtTSAR2* might also shift metabolite flux from steroidal to triterpene saponin biosynthesis in fenugreek. We therefore transformed fenugreek hairy roots with the *MtTSAR1* and *MtTSAR2* coding sequences from *Medicago truncatula* cloned independently under the control of the *LjUBQ1* promoter (*pUB::TSAR1/2::Hyg*, Supporting Fig. S1) and used the corresponding empty T-DNA as a control. Transformed roots were identified by PCR targeting a fragment of the hygromycin resistance gene present in these constructs (Supporting Fig. S6). Roots identified in this manner were screened for expression of *MtTSAR1* or *MtTSAR2* using qPCR, and multiple individually transformed roots expressing these transcription factors were identified (Fig. 4A and B). As expected, there was no detectable expression of either *MtTSAR1* or *MtTSAR2* in control samples. However, 66.7% (8/12 plants) of *MtTSAR1*-transformed plants and

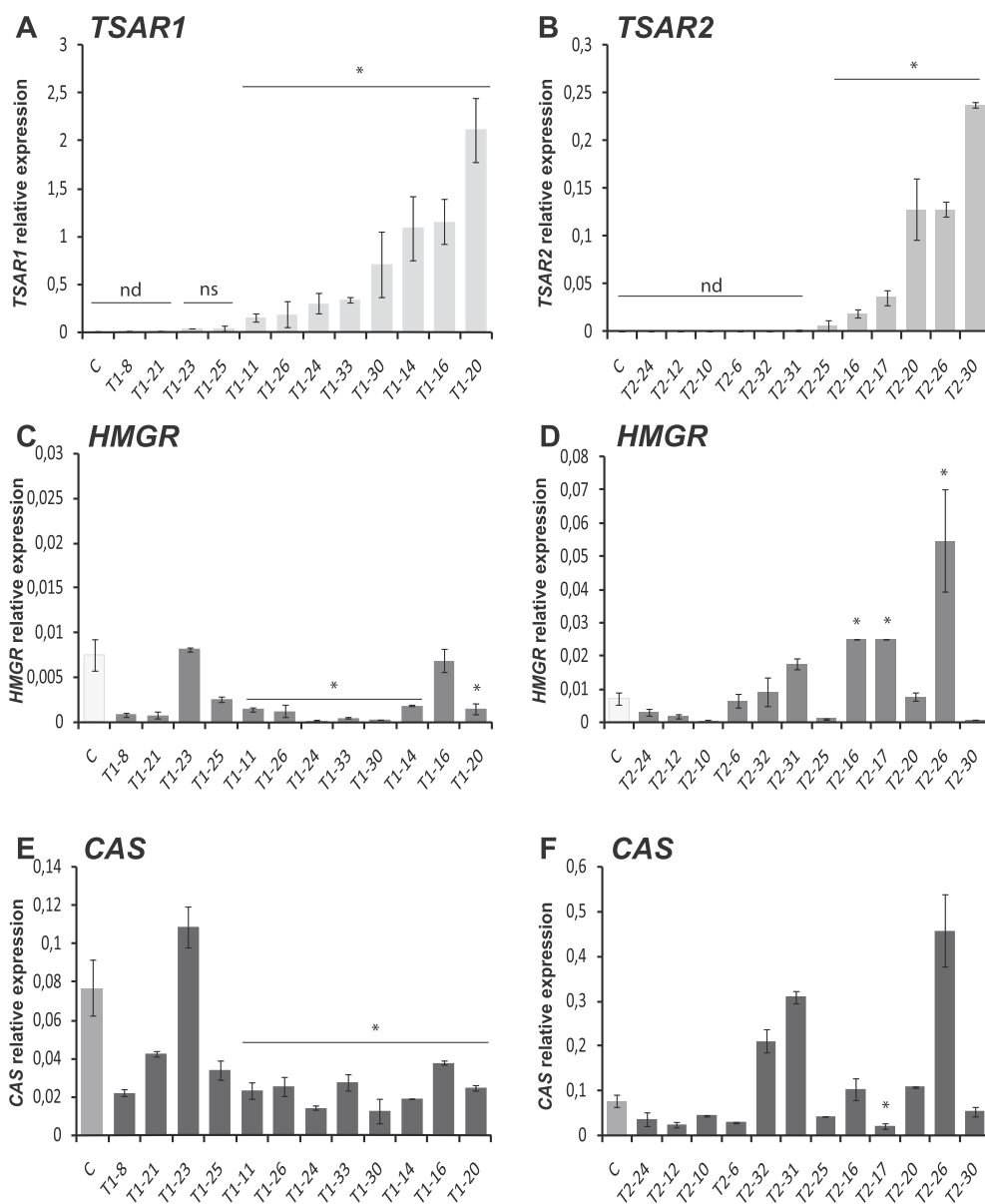


Fig. 4. Heterologous expression of *MtTSAR1* and *MtTSAR2* in fenugreek hairy roots and the effect on HMGR and CAS transcript levels. Relative transcript expression estimated by qRT-PCR in control (empty vector) compared to A, C, and E) *TSAR1*-expressing and B, D, and F) *TSAR2*-expressing hairy roots; A) *TSAR1* expression; B) *TSAR2* expression; C and D) *HMGR* expression in *TSAR1* and *TSAR2* lines, respectively; E and F) *CAS* expression in *TSAR1* and *TSAR2* lines, respectively; Values shown are the average \pm SD of two technical replicates for each individual hairy root. Values for the control are the average \pm SD of two technical replicates for each of three biological replicates ($n = 3$). *denotes significant change compared to the corresponding controls only in *TSAR1*-or *TSAR2*-expressing roots, “nd” = not detected, “ns” = not significant.

42% (5/12 plants) of *MtTSAR2* transformed plants showed heterologous expression of the corresponding *TSAR1* and *TSAR2* genes compared to the control samples (Fig. 4A and B, asterisks).

Since we confirmed heterologous expression of *MtTSAR1* and *MtTSAR2* in fenugreek, the question arose whether these transcripts are eventually translated into functional proteins, *i.e.* active transcription factors that are capable of regulating the expression of genes involved in the triterpene/sterol biosynthesis pathway in fenugreek. Thus, we tested whether *MtTSAR1* and *MtTSAR2* could alter the expression of saponin biosynthetic genes in fenugreek, as they do in *Medicago*, by using two key genes controlling flux control and branch points in the saponin biosynthesis pathway: The first, encoding 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGR), which constitutes an early point regulating carbon flux towards the triterpene and sterol biosynthesis pathways, and the second, cycloartenol synthase (CAS), which diverts the common biosynthetic precursor for steroidal and triterpene saponin biosynthesis towards sterol production (Mertens et al., 2016a; Moses et al., 2014; Sonawane et al., 2016). We found that 87.5% (7/8) of plant lines expressing *MtTSAR1* (Fig. 4C) have reduced *HMGR* expression levels. Interestingly, 50% of lines (3/6) expressing the *MtTSAR2* transcription factor (Fig. 4D) showed increased *HMGR*

expression, while in the remaining lines this was unchanged or reduced compared to controls. This finding indicates a difference in ability of the *MtTSARs* to affect fenugreek endogenous gene expression. Both *MtTSAR1* and *MtTSAR2* strongly suppress *CAS* transcript expression in *Medicago* (Mertens et al., 2016a). This was also the case for all of the *MtTSAR1*-expressing fenugreek lines, in which *CAS* expression was strongly suppressed (Fig. 4E). However, *MtTSAR2* had a minimal effect on fenugreek *CAS* expression with only 1/6 hairy root lines showing reduced *CAS* expression (Fig. 4F). Additionally, *MtTSAR1* and *MtTSAR2* heterologous expression levels do not correlate either with *HMGR* or *CAS* (Supporting Table S3A).

Because *CAS* expression was reduced in many of the *TSAR1*-expressing hairy roots, we tested whether there was a corresponding change in the saponin content in these lines, similar to that reported for *Medicago* hairy root lines overexpressing either one of the *TSARs*. Extracted saponins were analysed by UHPLC-MS/MS, but no consistent modulation of putative saponins was observed (Fig. 5), nor was there an observed increase in any saponin-like compounds identified during this analysis (not shown). Thus, while the heterologous expression of *MtTSAR1* and *MtTSAR2* in fenugreek does alter the transcription of endogenous saponin biosynthesis genes, we did not observe a major

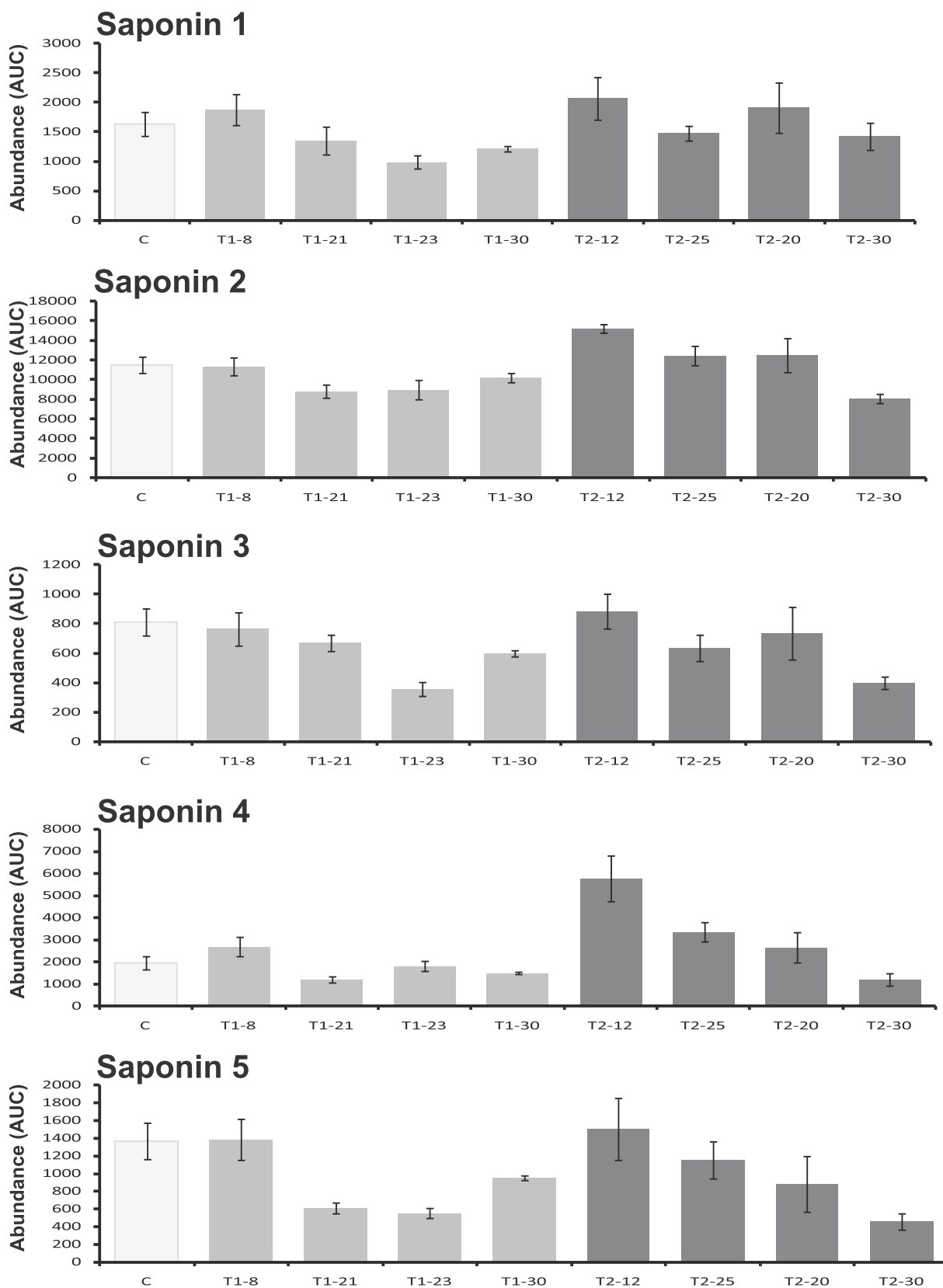


Fig. 5. Relative abundance of five representative saponins in Fenugreek hairy roots expressing the heterologous *MtTSAR1* and *MtTSAR2* transcription factors. Values shown are the average \pm SEM of at least three technical replicates per individual line. Values for the control are the average \pm SEM of at least three technical replicates for each of three biological replicates ($n = 3$). No significant differences were found using One-way ANOVA.

impact on actual saponin content either as a decrease in steroidal saponins or an increase in triterpene saponins.

To further probe the possible heterologous activity of the *MtTSARs* in fenugreek we transformed *Lotus japonicus* hairy roots with the same *MtTSAR1* and *MtTSAR2* expression constructs (Supporting Figs. S7A and B). Interestingly, *MtTSAR1* did not affect *LjHMGR2* and only induced *LjHMGR1* and *LjHMGR3* in one of three lines (Supporting Figs. S7C and D). However, *MtTSAR1* expression showed a significant positive correlation with *LjHMGR1* and *LjHMGR3* expression levels. On the other hand, plants expressing *MtTSAR2* showed a consistent induction of one out of three endogenous *Lotus HMGR* genes, *LjHMGR2*, compared to empty-vector transformed control lines, although there was no significant correlation of *MtTSAR2* with any of the *LjHMGRs* (Supporting Table S3B). This suggests that the *MtTSARs* are able to influence endogenous gene transcription in other legumes in addition to *Medicago*.

4. Discussion

Use of hairy root cultures leads to greater production of important products and secondary metabolites of plants in higher yields compared to the normal, non-transformed plants (Chen and Otten, 2017; Nagella et al., 2013). Developing and scaling up the efficiency of hairy root transformation in each plant species requires the evaluation and determination of many critical factors, such as the *Agrobacterium* strain used, the inoculum size, growth medium and antibiotic selection conditions (Srivastava and Srivastava, 2007). We have successfully developed a reproducible hairy root transformation method for fenugreek, which we envision being utilised both to explore the biosynthetic pathways underlying this plant's rich specialised metabolite profile, and as a platform for production of specific metabolites.

Two previous works discuss the induction of hairy roots and transformation of fenugreek using *Agrobacterium rhizogenes*. In one instance, the transformation rate and efficiency of root regeneration was not reported (Merkli et al., 1997). In the second case (Shahabzadeh et al., 2014), researchers used a particularly aggressive agropine-type *A. rhizogenes* strain K599 (Cho et al., 2000) to achieve a reported transformation efficiency between 70 and 80% using the “stem pricking” method and successful transformation was validated by PCR. Successful detection of 35S-driven GFP fluorescence was reported, contrary to our results, which showed that multiple 35S-promoter based constructs did not lead to strong transgene overexpression in fenugreek. Crucially, neither of these methods has been used in subsequent literature. Our method, based on a previous protocol for *Medicago truncatula* (Boisson-Dernier et al., 2001) yielded a lower, yet perfectly sufficient, transformation efficiency of ~50% using the less aggressive agropine ARqua1 *A. rhizogenes* strain. Moreover, we have demonstrated robust transgene expression using the *LjUBQ* promoter and multiple heterologous coding sequences.

Ideally, because hairy root transformations are by nature transient, and require aseptic clonal propagation of developed lines, a protocol for stable transformation could be developed for fenugreek in order to further facilitate both basic research and metabolic engineering applications. One attempt to date showed successful induction of fenugreek shoots from dedifferentiated callus cells, but screening a range of different hormone types, amounts, and combinations thereof failed to induce root regeneration (Aasim et al., 2010). Conceivably, this approach to shoot regeneration coupled with our proposed method of callus and hairy root induction using *A. rhizogenes* could be adapted for the generation of transgenic fenugreek lines, similar to the approach developed for *Lotus japonicus* (Stiller et al., 1997).

In our experiments, the *LjUBQ1* promoter is far more robust than CaMV35S in fenugreek roots, based on: the frequency and intensity of observed GFP fluorescence, the inability of a previously validated (Maekawa et al., 2008) 35S-driven hygromycin resistance gene to confer hygromycin resistance to transformed hairy roots, and the lack

of betalain pigment production in plants transformed with a validated vector encoding betalain biosynthetic genes under control of the 35S promoter (Polturak et al., 2016). This indicates very low activity of the CaMV35S promoter in fenugreek, similar to *Lotus* (Maekawa et al., 2008) and rice (Jang et al., 2002; Cornejo et al., 1993). Promoters can preserve their native functionality in transgenic plants, but qualitative and quantitative variations in transgene expression can be observed. Constitutive promoters typically originate from either plant viruses or plant housekeeping genes (Hernandez Garcia et al., 2014). CaMV35S is undoubtedly one of the most common promoters used (Odell et al., 1985), but does not function equally well in all species (Wilmlink et al., 1995), and moreover is reportedly silenced in sections of nodules and conceivably other tissues in *Medicago truncatula* (Auriac and Timmers, 2007). Thus the identification of other promoters is crucial in expanding transgenic-based genome editing and metabolic engineering strategies to new species and in particular to legumes.

Over-expression of the *MtTSAR1* and *MtTSAR2* transcription factors in *Medicago* resulted in changes in saponin biosynthetic gene expression levels: at early stages of the biosynthesis pathway up to the production of 2,3-oxidosqualene, at the stage of 2,3-oxidosqualene cyclisation to β -amyrin, and at the downstream oxidation and glycosylation steps (Mertens et al., 2016a). This correspondingly led to an increase in hemolytic or non-hemolytic triterpene saponin levels, depending on which TSAR transcription factor was overexpressed (Mertens et al., 2016a). A similar alteration in saponin biosynthesis gene transcript levels and an increase in non-hemolytic saponin accumulation was observed when the *CrBIS1* (bHLH irridoid synthesis 1) from *Catharanthus roseus* was heterologously expressed in *Medicago* hairy roots (Mertens et al., 2016b). We did not observe a significant alteration in saponins in fenugreek roots expressing *MtTSAR1* and *MtTSAR2*. Conceivably, since regenerated hairy roots were grown without selection, some of those roots identified as transformed based on PCR are mosaic, similar to those transformed with GFP (Fig. 3A, arrowheads). These mosaic roots will show TSAR transcript accumulation greater than control roots (in which they are non-detectable, Fig. 4A and B), but this accumulation in only a subset of root cells might not translate to a significant alteration in metabolite abundances.

One of the genes consistently induced by *MtTSAR1*, *MtTSAR2* and *CrBIS1* expression in *M. truncatula* hairy roots was the *MtHMGR1* gene (Mertens et al., 2016a, 2016b). When we expressed *MtTSAR1* in fenugreek we observed a decrease in *HMGR* transcript levels in almost all roots. However, we observed a clear induction of *HMGR* transcription by heterologous expression of *MtTSAR2* in fenugreek. This was comparable to the effects of heterologous *MtTSAR1* and *MtTSAR2* expression in *Lotus japonicus* hairy roots, wherein *MtTSAR2* consistently induced the expression levels of the endogenous *LjHMGR2* gene, while *MtTSAR1* caused an induction of *LjHMGR1* and *LjHMGR3* in only one of three roots (Supporting Fig. S7). In spite of this effect, in none of these cases did this result in a corresponding change in triterpene or steroidal saponins or their precursor aglycone levels (not shown).

A similar effect to what we found with the *MtTSARs* in fenugreek and *Lotus* has already been observed in tobacco, where the heterologous expression of two transcription factors (Myb308 and Myb305) from *Antirrhinum* caused the inhibition of phenolic acid and lignin biosynthesis (Tamagnone et al., 1998). However, why this happens remains unclear. It was suggested that it is a result of competition for DNA binding between endogenous and heterologous transcription factors, with the latter having weaker binding affinity for the corresponding regulatory motifs in heterologous species genes. The induction of triterpene saponin biosynthetic genes by the TSARs in *Medicago* was dependent on the presence of conserved N-box motifs within the first 500bp upstream of the start codon in the target genes promoter regions (Mertens et al., 2016a). A full genome sequence is not publicly available for fenugreek, but it is interesting to note that in *Lotus*, only the *LjHMGR2* promoter sequence, which we found to be induced by heterologous expression of *MtTSAR2*, contains an N-box motif less than

500 bp upstream of the start codon. On the contrary, *LjHMGR1*, which contains an N-box ~1 kb upstream of its start codon, and *LjHMGR3*, which contains none, were largely unaffected by both *MtTSAR1* and *MtTSAR2* expression (Supporting Table S4). Conceivably, fenugreek and Lotus have evolved different transcriptional regulation networks for control of their triterpene or steroidal saponin biosynthesis genes, or they have evolved different transcription factor-promoter binding characteristics.

Alternatively, given the high expression levels of the heterologous TSAR transcription factors, even weak interactions at DNA binding sites could significantly inhibit the binding activity and function of endogenous transcription factors in fenugreek and Lotus. Also, direct protein interaction between transcription factors has been demonstrated (Jackson, 1992). Conserved regions of the basic helix-loop-helix (bHLH) structure of TSARs, which are normally responsible for interaction with other proteins (Ambawat et al., 2013; Feller et al., 2011), could lead to a non-functional or aberrant interaction with endogenous transcription factors in the transformed fenugreek roots. This is a plausible scenario, as both Lotus and fenugreek express transcripts that are phylogenetically close to *MtTSAR1* and *MtTSAR2* (Supporting Fig. S8) and share 50–65% sequence identity with their corresponding Medicago TSAR protein (Supporting Table S5).

In conclusion, we have developed a robust and reproducible method for the transformation of fenugreek hairy roots and have identified a promoter suitable for strong constitutive expression of transgenes in this system. We have also shown that the regulation of saponin metabolism retains sufficient similarity between legumes, so that the heterologous expression of transcription factors between them affects the expression of endogenous genes, but with reduced efficiency even in species belonging to the same phylogenetic family. This requires further clarification if regulatory components of plant specialised metabolism are to be efficiently exploited in heterologous systems for metabolic engineering. We thus demonstrate that a similar comparative strategy to that we employed, combined with the data derived from transcriptomic and potentially genomic databases for fenugreek, can be used in order to develop this system and other biochemically-rich non-model plants into suitable resources for novel metabolite mining and chassis development for metabolic engineering approaches.

Contributions

CG and KKP conceived the study and designed and coordinated experimental efforts. CG, KB, MEG conducted experiments, analysed data and prepared figures. PDS provided technical expertise and carried out metabolite analysis. KH provided resources and helped conceive experiments. AG provided TSAR clones and facilitated analysis of samples from Lotus. AA provided analytical equipment for fenugreek metabolite analysis and resources. CG and KKP wrote the paper. All authors received and approved the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Irene Chionidou and Fotini Dimakou for assistance with fenugreek and Lotus hairy root transformations. We acknowledge support of this work by the project: “Synthetic Biology: from omics technologies to genomic engineering” (OMIC-ENGINE) (MIS 5002636), which is implemented under the Action Reinforcement of the Research and Innovation Infrastructure, funded by the Operational Programme Competitiveness, Entrepreneurship, and

Innovation (NSRF 2014–2020) and co-financed by Greece and the European Union (European Regional Development Fund) (to KKP), and by the European Community's Seventh Framework Program (FP7/2007–2013) under grant agreement 613692 (TriForC) (AG, AA, KKP). CG was supported by an EMBO short-term fellowship, number 8090.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2020.06.011>.

References

- Aasim, M., Hussain, N., Umer, E.M., Zubair, M., Bilal, S., Saeed, S., Rafique, T.S., Sancak, C., 2010. In vitro shoot regeneration of fenugreek (*Trigonella foenum-graecum* L.) using different cytokinins. *Afr. J. Biotechnol.* 9, 7174–7179.
- Acharya, S.N., Thomas, J.E., Basu, S.K., 2008. Fenugreek, an Alternative Crop for Semiarid Regions of North America. vol. 48.
- Aggarwal, P.R., Nag, P., Choudhary, P., Chakraborty, N., Chakraborty, S., 2018. Genotype-independent *Agrobacterium* rhizogenes-mediated root transformation of chickpea: a rapid and efficient method for reverse genetics studies. *Plant Methods* 14, 55.
- Altuntaş, E., Özgöz, E., Taşer, Ö.F., 2005. Some physical properties of fenugreek (*Trigonella foenum-graecum* L.) seeds. *J. Food Eng.* 71, 37–43.
- Ambawat, S., Sharma, P., Yadav, N.R., Yadav, R.C., 2013. MYB transcription factor genes as regulators for plant responses: an overview. *Physiol. Mol. Biol. Plants* 19, 307–321.
- Anisimova, M., Gascuel, O., 2006. Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. *Syst. Biol.* 55, 539–552.
- Antonovsky, N., Gleizer, S., Noor, E., Zohar, Y., Herz, E., Barenholz, U., Zelcbuch, L., Amram, S., Wides, A., Tepper, N., Davidi, D., Bar-On, Y., Bareia, T., Wernick, D.G., Shani, I., Malitsky, S., Jona, G., Bar-Even, A., Milo, R., 2016. Sugar synthesis from CO₂ in *Escherichia coli*. *Cell* 166, 115–125.
- Arendt, P., Miettinen, K., Pollier, J., De Rycke, R., Callewaert, N., Goossens, A., 2017. An endoplasmic reticulum-engineered yeast platform for overproduction of triterpenoids. *Metab. Eng.* 40, 165–175.
- Auriac, M.-C., Timmers, A.C.J., 2007. Nodulation studies in the model legume *Medicago truncatula*: advantages of using the constitutive EF1 α promoter and limitations in detecting fluorescent reporter proteins in nodule tissues. *Mol. Plant Microbe Interact.* 20, 1040–1047.
- Belguith-Hadri, O., Bouaziz, M., Jamoussi, K., Simmonds, M.S., El Feki, A., Makni-Ayedi, F., 2013. Comparative study on hypocholesterolemic and antioxidant activities of various extracts of fenugreek seeds. *Food Chem.* 138, 1448–1453.
- Boisson-Dernier, A., Chabaud, M., Garcia, F., Becard, G., Rosenberg, C., Barker, D.G., 2001. *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. *Mol. Plant Microbe Interact.* 14, 695–700.
- Bonaldi, K., Gherbi, H., Franche, C., Bastien, G., Fardoux, J., Barker, D., Giraud, E., Cartieaux, F., 2010. The Nod factor-independent symbiotic signaling pathway: development of *Agrobacterium rhizogenes*-mediated transformation for the legume *Aeschynomene indica*. *Mol. Plant-Microbe Interact. : MPMI (Mol. Plant-Microbe Interact.)* 23, 1537–1544.
- Cardenas, P.D., Sonawane, P.D., Pollier, J., Vanden Bossche, R., Dewangan, V., Weithorn, E., Tal, L., Meir, S., Rogachev, I., Malitsky, S., Giri, A.P., Goossens, A., Burdman, S., Aharoni, A., 2016. GAME9 regulates the biosynthesis of steroidal alkaloids and upstream isoprenoids in the plant mevalonate pathway. *Nat. Commun.* 7, 10654.
- Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* 17, 540–552.
- Chaudhary, S., Chikara, S.K., Sharma, M.C., Chaudhary, A., Alam Syed, B., Chaudhary, P.S., Mehta, A., Patel, M., Ghosh, A., Iriti, M., 2015. Elicitation of diosgenin production in *trigonella foenum-graecum* (fenugreek) seedlings by methyl jasmonate. *Int. J. Mol. Sci.* 16, 29889–29899.
- Chen, K., Otten, L., 2017. Natural *Agrobacterium* transformants: recent results and some theoretical considerations. *Front. Plant Sci.* 8.
- Chen, P.S., Shih, Y.W., Huang, H.C., Cheng, H.W., 2011. Diosgenin, a steroidal saponin, inhibits migration and invasion of human prostate cancer PC-3 cells by reducing matrix metalloproteinases expression. *PLoS One* 6, e20164.
- Chevenet, F., Brun, C., Banuls, A.L., Jacq, B., Christen, R., 2006. TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC Bioinf.* 7, 439.
- Chiang, C.T., Way, T.D., Tsai, S.J., Lin, J.K., 2007. Diosgenin, a naturally occurring steroid, suppresses fatty acid synthase expression in HER2-overexpressing breast cancer cells through modulating Akt, mTOR and JNK phosphorylation. *FEBS Lett.* 581, 5735–5742.
- Chilton, M.-D., Tepfer, D.A., Petit, A., David, C., Casse-Delbart, F., Tempé, J., 1982. *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. *Nature* 295, 432.
- Chiu, W.-L., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H., Sheen, J., 1996. Engineered GFP as a vital reporter in plants. *Curr. Biol.* 6, 325–330.
- Cho, H., Farrand, S.K., Noel, G.R., Widholm, J.M., 2000. High efficiency induction of soybean hairy roots and propagation of the soybean cyst nematode. *Planta* 210, 195–204.
- Christ, B., Xu, C., Xu, M., Li, F.S., Wada, N., Mitchell, A.J., Han, X.L., Wen, M.L., Fujita, M., Weng, J.K., 2019. Repeated evolution of cytochrome P450-mediated spiroketal

- steroid biosynthesis in plants. *Nat. Commun.* 10, 3206.
- Ciura, J., Szeliga, M., Grzesik, M., Tyrka, M., 2017. Changes in Fenugreek Transcriptome Induced by Methyl Jasmonate and Steroid Precursors Revealed by RNA-Seq Genomics.
- Clemow, S.R., Clairmont, L., Madsen, L.H., Guinel, F.C., 2011. Reproducible hairy root transformation and spot-inoculation methods to study root symbioses of pea. *Plant Methods* 7, 46.
- Cornejo, M.J., Luth, D., Blankenship, K.M., Anderson, O.D., Blechl, A.E., 1993. Activity of a maize ubiquitin promoter in transgenic rice. *Plant Mol. Biol.* 23, 567–581.
- Delis, C., Krokida, A., Georgiou, S., Pena-Rodriguez, L.M., Kavroulakis, N., Ioannou, E., Roussis, V., Osbourn, A.E., Papadopoulou, K.K., 2011. Role of lipoel synthase in *Lotus japonicus* nodule formation. *New Phytol.* 189, 335–346.
- Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.M., Gascuel, O., 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res.* 36, W465–W469.
- Dereeper, A., Audic, S., Claverie, J.M., Blanc, G., 2010. BLAST-EXPLORER helps you building datasets for phylogenetic analysis. *BMC Evol. Biol.* 10, 8.
- Díaz, C.L., Melchers, L.S., Hooykaas, P.J.J., Lugtenberg, B.J.J., Kijne, J.W., 1989. Root lectin as a determinant of host-plant specificity in the Rhizobium-legume symbiosis. *Nature* 338, 579–581.
- Díaz, C.L., Spaink, H.P., Kijne, J.W., 2000. Heterologous rhizobial lipochitin oligosaccharides and chitin oligomers induce cortical cell divisions in red clover roots, transformed with the pea lectin gene. *Mol. Plant-Microbe Interact.: MPMI (Mol. Plant-Microbe Interact.)* 13, 268–276.
- Diouf, D., Gherbi, H., Prin, Y., Franche, C., Duhoux, E., Bogusz, D., 1995. Hairy root nodulation of *Casuarina glauca*: a system for the study of symbiotic gene expression in an actinorhizal tree, Molecular plant-microbe interactions. *MPMI (Mol. Plant-Microbe Interact.)* 8, 532–537.
- Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797.
- El Nasri, N.A., El Tinay, A.H., 2007. Functional properties of fenugreek (*Trigonella foenum graecum*) protein concentrate. *Food Chem.* 103, 582–589.
- Engler, C., Youles, M., Gruetznher, R., Ehner, T.M., Werner, S., Jones, J.D., Patron, N.J., Marillonnet, S., 2014. A golden gate modular cloning toolbox for plants. *ACS Synth. Biol.* 3, 839–843.
- Estrada-Navarrete, G., Alvarado-Affantranger, X., Olivares, J.E., Guillen, G., Diaz-Camino, C., Campos, F., Quinto, C., Gresshoff, P.M., Sanchez, F., 2007. Fast, efficient and reproducible genetic transformation of *Phaseolus* spp. by *Agrobacterium rhizogenes*. *Nat. Protoc.* 2, 1819–1824.
- Estruch, J.J., Chriqui, D., Grossmann, K., Schell, J., Spena, A., 1991. The plant oncogene *rolC* is responsible for the release of cytokinins from glucoside conjugates. *EMBO J.* 10, 2889–2895.
- Fedacko, J., Singh, R., Niaz, M.A., Ghosh, S., Fedackova, P., Tripathi, A.D., Etharat, A., Onsaard, E., Singh, V.K., Shastun, S., 2016. Fenugreek seeds decrease blood cholesterol and blood glucose as adjunct to diet therapy in patients with hypercholesterolemia. *World Heart J.* 8 (3), 239–249.
- Feller, A., Macheiner, K., Braun, E.L., Grotewold, E., 2011. Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. *Plant J. : Cell Mol. Biol.* 66, 94–116.
- Flammang, A.M., Cifone, M.A., Erexson, G.L., Stankowski Jr., L.F., 2004. Genotoxicity testing of a fenugreek extract. *Food Chem. Toxicol. : Int. J. Pub. Br. Biol. Res. Assoc.* 42, 1769–1775.
- Gaddam, A., Galla, C., Thummiseti, S., Marikanty, R.K., Palanisamy, U.D., Rao, P.V., 2015. Role of Fenugreek in the prevention of type 2 diabetes mellitus in prediabetes. *J. Diabetes Metab. Disord.* 14, 74.
- Gleizer, S., Ben-Nissan, R., Bar-On, Y.M., Antonovsky, N., Noor, E., Zohar, Y., Jona, G., Krieger, E., Shamshoum, M., Bar-Even, A., Milo, R., 2019. Conversion of *Escherichia coli* to generate all biomass carbon from CO₂. *Cell* 179, 1255–1263 e1212.
- Gomes, C., Dupas, A., Pagano, A., Grima-Pettenati, J., Paiva, J.A.P., 2019. Hairy root transformation: a useful tool to explore gene function and expression in salix spp. recalcitrant to transformation. *Front. Plant Sci.* 10, 1427.
- Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* 52, 696–704.
- Hooykaas, P.J.J., Klapwijk, P.M., Nuti, M.P., Schilperoord, R.A., Rorsch, A., 1977. Transfer of the *Agrobacterium tumefaciens* TI plasmid to avirulent *Agrobacteria* and to *Rhizobium* *ex planta*. *J. Gen. Microbiol.* 98, 477–484.
- Imanishi, L., Vayssières, A., Franche, C., Bogusz, D., Wall, L., Svistoonoff, S., 2011. Transformed hairy roots of *Discaria trinervis*: a valuable tool for studying actinorhizal symbiosis in the context of intercellular infection. *Mol. Plant-Microbe Interact.: MPMI (Mol. Plant-Microbe Interact.)* 24, 1317–1324.
- Jackson, S.P., 1992. Regulating transcription factor activity by phosphorylation. *Trends Cell Biol.* 2, 104–108.
- Jaiswal, N., Maurya, C.K., Venkateswarlu, K., Sukanya, P., Srivastava, A.K., Narender, T., Tamrakar, A.K., 2012. 4-Hydroxyisoleucine stimulates glucose uptake by increasing surface GLUT4 level in skeletal muscle cells via phosphatidylinositol-3-kinase-dependent pathway. *Eur. J. Nutr.* 51, 893–898.
- Jang, I.C., Choi, W.B., Lee, K.H., Song, S.I., Nahm, B.H., Kim, J.K., 2002. High-level and ubiquitous expression of the rice cytochrome c gene *OsCc1* and its promoter activity in transgenic plants provides a useful promoter for transgenesis of monocots. *Plant Physiol.* 129, 1473–1481.
- Jani, R., Udipi, S.A., Ghugre, P.S., 2009. Mineral content of complementary foods. *Indian J. Pediatr.* 76.
- Kan, J., Velliquette, R.A., Grann, K., Burns, C.R., Scholten, J., Tian, F., Zhang, Q., Gui, M., 2017. A novel botanical formula prevents diabetes by improving insulin resistance. *BMC Compl. Alternative Med.* 17, 352.
- Kang, L.P., Zhao, Y., Pang, X., Yu, H.S., Xiong, C.Q., Zhang, J., Gao, Y., Yu, K., Liu, C., Ma, B.P., 2013. Characterization and identification of steroidal saponins from the seeds of *Trigonella foenum-graecum* by ultra high-performance liquid chromatography and hybrid time-of-flight mass spectrometry. *J. Pharmaceut. Biomed. Anal.* 74, 257–267.
- Kenny, O., Smyth, T.J., Hewage, C.M., Brunton, N.P., 2013. Antioxidant properties and quantitative UPLC-MS analysis of phenolic compounds from extracts of fenugreek (*Trigonella foenum-graecum*) seeds and bitter melon (*Momordica charantia*) fruit. *Food Chem.* 141, 4295–4302.
- Kereszt, A., Li, D., Indrasumunar, A., Nguyen, C.D., Nontachaiyapoom, S., Kinkema, M., Gresshoff, P.M., 2007. *Agrobacterium rhizogenes*-mediated transformation of soybean to study root biology. *Nat. Protoc.* 2, 948–952.
- Khole, S., Chatterjee, S., Variyar, P., Sharma, A., Devasagayam, T.P.A., Ghaskadbi, S., 2014. Bioactive constituents of germinated fenugreek seeds with strong antioxidant potential. *J. Functional Foods* 6, 270–279.
- Knott, E.J., Richard, A.J., Mynatt, R.L., Ribnicky, D., Stephens, J.M., Bruce-Keller, A., 2017. Fenugreek supplementation during high-fat feeding improves specific markers of metabolic health. *Sci. Rep.* 7, 12770.
- Krokida, A., Delis, C., Geisler, K., Garagounis, C., Tsikou, D., Pena-Rodriguez, L.M., Katsarou, D., Field, B., Osbourn, A.E., Papadopoulou, K.K., 2013. A metabolic gene cluster in *Lotus japonicus* discloses novel enzyme functions and products in triterpene biosynthesis. *New Phytol.* 200, 675–690.
- Kung, S.H., Lund, S., Murarka, A., McPhee, D., Paddon, C.J., 2018. Approaches and recent developments for the commercial production of semi-synthetic artemisinin. *Front. Plant Sci.* 9, 87.
- Lau, W., Fischbach, M.A., Osbourn, A., Sattely, E.S., 2014. Key applications of plant metabolic engineering. *PLoS Biol.* 12, e1001879.
- Lee, N.G., Stein, B., Suzuki, H., Verma, D.P., 1993. Expression of antisense nodulin-35 RNA in *Vigna acutifolia* transgenic root nodules retards peroxisome development and affects nitrogen availability to the plant. *Plant J. : Cell Mol. Biol.* 3, 599–606.
- Li, F., Fernandez, P.P., Rajendran, P., Hui, K.M., Sethi, G., 2010. Diosgenin, a steroidal saponin, inhibits STAT3 signaling pathway leading to suppression of proliferation and chemosensitization of human hepatocellular carcinoma cells. *Canc. Lett.* 292, 197–207.
- Limpens, E., Ramos, J., Franken, C., Raz, V., Compaan, B., Franssen, H., Bisseling, T., Geurts, R., 2004. RNA interference in *Agrobacterium rhizogenes*-transformed roots of *Arabidopsis* and *Medicago truncatula*. *J. Exp. Bot.* 55, 983–992.
- Lu, X., Tang, K., Li, P., 2016. Plant metabolic engineering strategies for the production of pharmaceutical terpenoids. *Front. Plant Sci.* 7, 1647.
- Maekawa, T., Kusakabe, M., Shimoda, Y., Sato, S., Tabata, S., Murooka, Y., Hayashi, M., 2008. Polyubiquitin promoter-based binary vectors for overexpression and gene silencing in *Lotus japonicus*. *Mol. Plant Pathogen Inter.* 21, 375–382.
- Mehrafarin, A., Qaderi, A., Rezazadeh, S., Naghdi Badi, H., Noormohammadi, G., Zand, E., 2010. Bioengineering of important secondary metabolites and metabolic pathways in fenugreek (*Trigonella foenum-graecum* L.). *J. Med. Plants* 9 (31).
- Mellor, K.E., Hoffman, A.M., Timko, M.P., 2012. Use of *ex vitro* composite plants to study the interaction of cowpea (*Vigna unguiculata* L.) with the root parasitic angiosperm *Striga gesnerioides*. *Plant Methods* 8, 22–22.
- Meng, D., Yang, Q., Dong, B., Song, Z., Niu, L., Wang, L., Cao, H., Li, H., Fu, Y., 2019. Development of an efficient root transgenic system for pigeon pea and its application to other important economically plants. *Plant Biotechnol. J.* 17, 1804–1813.
- Merkli, A., Christen, P., Kapetanidis, I., 1997. Production of diosgenin by hairy root cultures of *Trigonella foenum-graecum* L. *Plant Cell Rep.* 16, 632–636.
- Mertens, J., Pollier, J., Vanden Bossche, R., Lopez-Vidriero, I., Franco-Zorrilla, J.M., Goossens, A., 2016a. The bHLH transcription factors TSAR1 and TSAR2 regulate triterpene saponin biosynthesis in *Medicago truncatula*. *Plant Physiol.* 170, 194–210.
- Mertens, J., Van Moerkercke, A., Vanden Bossche, R., Pollier, J., Goossens, A., 2016b. Clade IVa basic helix-loop-helix transcription factors form part of a conserved jasmonate signaling circuit for the regulation of bioactive plant terpenoid biosynthesis. *Plant Cell Physiol.* 57, 2564–2575.
- Moses, T., Papadopoulou, K.K., Osbourn, A., 2014. Metabolic and functional diversity of saponins, biosynthetic intermediates and semi-synthetic derivatives. *Crit. Rev. Biochem. Mol. Biol.* 49, 439–462.
- Nagella, P., Thiruvengadam, M., Jung, S.J., Murthy, H.N., Chung, I.M., 2013. Establishment of *Gymnema sylvestre* hairy root cultures for the production of gymnemic acid. *Acta Physiol. Plant.* 35, 3067–3073.
- Nikel, P.I., de Lorenzo, V., 2018. *Pseudomonas putida* as a functional chassis for industrial biocatalysis: from native biochemistry to trans-metabolism. *Metab. Eng.* 50, 142–155.
- Niwa, Y., Hirano, T., Yoshimoto, K., Shimizu, M., Kobayashi, H., 1999. Non-invasive quantitative detection and applications of non-toxic, S65T-type green fluorescent protein in living plants. *Plant J.* 18, 455–463.
- Norziah, M.H., Fezea, F.A., Bhat, R., Ahmad, M., 2015. Effect of extraction solvents on antioxidant and antimicrobial properties of fenugreek seeds (*Trigonella foenum-graecum*). *Int. Food Res. J.* 22, 1261–1271.
- Peraza-Luna, F., Rodríguez-Mendiola, M., Arias-Castro, C., Bessiere, J.-M., Calva-Calva, G., 2001. Sotolon production by hairy root cultures of *Trigonella foenum-graecum* in airlift with mesh bioreactors. *J. Agric. Food Chem.* 49, 6012–6019.
- Petropoulos, G., 2002. Fenugreek. Taylor and Francis, London.
- Polturak, G., Breitl, D., Grossman, N., Sarrion-Pradigones, A., Weithorn, E., Pliner, M., Orzaez, D., Granel, A., Rogachev, I., Aharoni, A., 2016. Elucidation of the first committed step in betalain biosynthesis enables the heterologous engineering of betalain pigments in plants. *New Phytol.* 210, 269–283.
- Pyne, M.E., Narcross, L., Martin, V.J.J., 2019. Engineering plant secondary metabolism in microbial systems. *Plant Physiol.* 179, 844–861.
- Quandt, H.J., 1993. Transgenic root nodules of *Vicia hirsuta*: a fast and efficient system for the study of gene expression in indeterminate-type nodules. *Mol. Plant-Microbe*

- Interact. 6, 699–706 1993 v.1996 no.1996.
- Rababah, T.M., Hettiarachchy, N.S., Horax, R., 2004. Total phenolics and antioxidant activities of fenugreek, green tea, black tea, grape seed, ginger, rosemary, gotu kola, and ginkgo extracts, vitamin E, and tert-butylhydroquinone. *J. Agric. Food Chem.* 52, 5183–5186.
- Raghuram, T.C., Sharma, R.D., Sivakumar, B., Sahay, B.K., 1994. Effect of fenugreek seeds on intravenous glucose disposition in non-insulin dependent diabetic patients. *Phytother Res.* 8, 83–86.
- Reed, J., Osbourn, A., 2018. Engineering terpenoid production through transient expression in *Nicotiana benthamiana*. *Plant Cell Rep.* 37, 1431–1441.
- Riker, A.J., Banfield, W.M., Wright, W.H., Keitt, W.H., Sagen, H.E., 1930. Studies on infectious hairy root of nursery apple trees. *J. Agric. Res.* 41, 507–540.
- Sarrion-Perdigones, A., Falconi, E.E., Zandalinas, S.I., Juarez, P., Fernandez-del-Carmen, A., Granell, A., Orzaez, D., 2011. GoldenBraid: an iterative cloning system for standardized assembly of reusable genetic modules. *PLoS One* 6, e21622.
- Sethi, G., Shanmugam, M.K., Warriar, S., Merarchi, M., Arfuso, F., Kumar, A.P., Bishayee, A., 2018. Pro-apoptotic and anti-cancer properties of diosgenin: a comprehensive and critical review. *Nutrients* 10.
- Shahabzadeh, Z., Heidari, B., Hafez, R.F., 2014. Induction of transgenic hairy roots in *Trigonella foenum-graecum* co-cultivated with *Agrobacterium rhizogenes* harboring a GFP gene. *J. Crop Sci. Biotechnol.* 16, 263–268.
- Sharma, V., Singh, P., Rani, A., 2017. Antimicrobial activity of *trigonella foenum-graecum* L. (Fenugreek). *Eur. J. Exp. Biol.* 7.
- Siefkes-Boer, H.J., Noonan, M.J., Bullock, D.W., Conner, A.J., 1995. Hairy root transformation system in large-seeded. *Grain Legumes* 43, 1.
- Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J.D., Higgins, D.G., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* 539.
- Sinharoy, S., Saha, S., Chaudhury, S.R., Dasgupta, M., 2009. Transformed hairy roots of *Arachis hypogea*: a tool for studying root nodule symbiosis in a non-infection thread legume of the Aeschynomeneae tribe. *Mol. Plant-Microbe Interact.: MPMI (Mol. Plant-Microbe Interact.)* 22, 132–142.
- Sinharoy, S., Pislariu, C.L., Udvardi, M.K., 2015. A High-Throughput RNA Interference (RNAi)-Based Approach Using Hairy Roots for the Study of Plant-Rhizobia Interactions. Springer.
- Skraly, F.A., Ambavaram, M.M.R., Peoples, O., Snell, K.D., 2018. Metabolic engineering to increase crop yield: from concept to execution. *Plant Sci. : Int. J. Exp. Plant Biol.* 273, 23–32.
- Sonawane, P.D., Pollier, J., Panda, S., Szymanski, J., Massalha, H., Yona, M., Unger, T., Malitsky, S., Arendt, P., Pauwels, L., Almekias-Siegl, E., Rogachev, I., Meir, S., Cardenas, P.D., Masri, A., Petrikov, M., Schaller, H., Schaffer, A.A., Kamble, A., Giri, A.P., Goossens, A., Aharoni, A., 2016. Plant cholesterol biosynthetic pathway overlaps with phytosterol metabolism. *Nature plants* 3, 16205.
- Spano, L., Mariotti, D., Pezzotti, M., Damiani, F., Arcioni, S., 1987. Hairy root transformation in alfalfa (*Medicago sativa* L.). *Theor. Appl. Genet.* 73, 523–530.
- Srinivasan, S., Koduru, S., Kumar, R., Venguswamy, G., Kyprianou, N., Damodaran, C., 2009. Diosgenin targets Akt-mediated prosurvival signaling in human breast cancer cells. *Int. J. Canc.* 125, 961–967.
- Srivastava, S., Srivastava, A.K., 2007. Hairy root culture for mass-production of high-value secondary metabolites. *Crit. Rev. Biotechnol.* 27, 29–43.
- Stiller, J., Martirani, L., Tuppare, S., Chian, R.-J., Chiurazzi, M., Gresshoff, P.M., 1997. High frequency transformation and regeneration of transgenic plants in the model legume *Lotus japonicus*. *J. Exp. Bot.* 48, 1357–1365.
- Stougaard, J., Petersen, T.E., Marcker, K.A., 1987. Expression of a complete soybean leghemoglobin gene in root nodules of transgenic *Lotus corniculatus*. *Proc. Natl. Acad. Sci. U. S. A.* 84, 5754–5757.
- Sukhapinda, K., Spivey, R., Shahin, E.A., 1987. Ri-plasmid as a helper for introducing vector DNA into alfalfa plants. *Plant Mol. Biol.* 8, 209–216.
- Szabo, K., Gesztelyi, R., Lampe, N., Kiss, R., Remenyik, J., Pesti-Asboth, G., Prikosz, D., Szilvassy, Z., Juhasz, B., 2018. Fenugreek (*trigonella foenum-graecum*) seed flour and diosgenin preserve endothelium-dependent arterial relaxation in a rat model of early-stage metabolic syndrome. *Int. J. Mol. Sci.* 19.
- Tamagnone, L., Merida, A., Parr, A., Mackay, S., Cullianez-Macia, F.A., Roberts, K., Martin, C., 1998. The AmMYB308 and AmMYB330 transcription factors from *Antirrhinum* regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. *Plant Cell* 10, 135.
- Timney, B.L., Raveh, B., Mironska, R., Trivedi, J.M., Kim, S.J., Russel, D., Wente, S.R., Sali, A., Rout, M.P., 2016. Simple rules for passive diffusion through the nuclear pore complex. *J. Cell Biol.* 215, 57–76.
- Wang, Y., Alonso, A.P., Wilkerson, C.G., Keegstra, K., 2012. Deep EST profiling of developing fenugreek endosperm to investigate galactomannan biosynthesis and its regulation. *Plant Mol. Biol.* 79, 243–258.
- Wilmink, A., van de Ven, B.C.E., Dons, J.J.M., 1995. Activity of constitutive promoters in various species from the Liliaceae. *Plant Mol. Biol.* 28, 949–955.
- Zandi, P., Basu, S.K., Khatibani, L.B., Balogun, M.O., Aremu, M.O., Sharma, M., Kumar, A., Sengupta, R., Li, X., Li, Y., Tashi, S., Hedi, A., Cetzal-Ix, W., 2014. Fenugreek (*Trigonella foenum-graecum* L.) seed: a review of physiological and biochemical properties and their genetic improvement. *Acta Physiol. Plant.* 37.