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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.

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

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Highlights

- A hairy-root transformation system for the crop and medicinal legume fenugreek was developed.
- *LjUbiquitin 1* promoter from *Lotus japonicus* leads to robust transgene expression in transformed fenugreek roots.
- Heterologous expression of Triterpene Saponin Activating Regulators from Medicago alters fenugreek terpenoid biosynthetic gene expression.

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.

Keywords

Fenugreek, *Trigonella foenum-graecum*, hairy root, *Agrobacterium rhizogenes* transformation, TSAR, Triterpene Saponin Activating Regulator;

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 specialized plant metabolites [1-3]. This expansion is aided by the advent of new high-throughput cloning methods [4, 5] and large-scale transcriptomic dataset acquisition, in conjunction with metabolic network modeling and prediction [6]. 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 [7-9], 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 [10, 11]. Seeds and leaves of the plant are widely used as forage, food and spice, but most importantly, for their medicinal properties [11, 12], which include: anti-diabetic [13-16], hypocholesterolemic [15, 16], antioxidant [17-20], anticancer, anti-metastatic [21-25], and anti-microbial [26, 27] activity. Major metabolic constituents of fenugreek, which contribute to its pharmaceutical traits, include 95 different steroidal and triterpene saponins and their precursor aglycones [28], notably the sterol diosgenin. Fenugreek also contains a plethora of: phenolics, alkaloids, fibers—specifically high amounts of galactomanans—vitamins and minerals [11, 17, 27, 29-37]. 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 specialized 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 [3] that can be used in parallel with heterologous transformation platforms, such as tobacco leaf infiltration [1] or introduction of plant

biosynthetic pathways into unicellular organisms [38-40]. The hairy root phenotype in plants is caused by a Gram-negative soil bacterium, *Agrobacterium rhizogenes*, which infects plants causing hairy root disease [41] by inserting a T-DNA derived from the root-inducing (*Ri*) plasmid into the plant genome [42] 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 [43, 44]. 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 [42, 45]. It is thus an important, low cost, simple and efficient system for genetic engineering established in many, often recalcitrant to transform, plant species [46, 47], including legumes [48-69]. Some works suggest that hairy root transformation is feasible in fenugreek [52, 70]. 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 [71], 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 (p*Lj*UBQ1) [72] 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* [73] 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 (H_2SO_4) for 7 minutes to aid imbibition (optionally, this step was omitted for fenugreek without a noticeable difference in germination), then washed 4 times with pre-chilled dH_2O and surface-sterilized with gentle shaking in a solution containing 20% (v/v) commercial bleach, 81 µM Tween-20 for 20 minutes. Sterile dH_2O was added and seeds were imbibed for 16 h under dark conditions in 4 ^{o}C. Seeds were placed onto 1% (w/v) agar in square petri dishes in a growth chamber (22^{o}C) under dark conditions for 24 hours. 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* [72], carrying a green fluorescent protein gene [eGFP S65T variant [74, 75]] 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 [72]. *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* [73]. 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 [76, 77] 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 ^{o}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 x 10 cm) dish. The dish was placed at an angle of approximately 45^{o} for 3-4 days in a 20 ^{o}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 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 Figure S1) did not confer resistance to fenugreek callus and hairy roots (Supporting Figure 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 ^{o}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: $MgSO_4$ 2 mM, KH_2PO_4 1 mM, FeEDTA 0.1 mM, KNO_3 10 mM, $Ca(NO_3)_2$ $4H_2O$ 5 mM, H_3BO_3 46 mM, $MnCl_2$ 9 mM, $ZnCl_2$ 0.8 mM, $CuCl_2$ 0.2 mM, H_2MoO_4 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 *Ds*Red2 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 [71] 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 [78]. The bHLH maximum likelihood phylogenetic tree was constructed on the phylogeny.fr server (http://www.phylogeny.fr/index.cgi) [79, 80] using MUSCLE [81] for alignment, GBLOCKs [82] to remove poorly aligned sequence blocks, PhyML [83, 84] for tree construction, and TreeDyn [85] 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 & Tobin, 1992). The expression levels of *TSAR1*, *TSAR2* [73] and predicted transcripts for fenugreek *HMGR* [86] and *CAS* [87] 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*) [36] 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 [76]. Two to three technical replicate reactions were carried out per sample. The reaction conditions were: reverse transcription at 55 ^{o}C for 10 min and then 40 cycles of 95 ^{o}C for 10 sec, 60 ^{o}C for 30 sec and melt-curve at 65-95 ^{o}C with increment 0.5 ^{o}C every 5 sec. Data analysis was performed according to the ΔC_T method (Livak & 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 composit 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 seconds, followed by

sonication in a bath sonicator for 20 min and again vortex for 30 seconds. Insoluble material was removed by centrifugation 20,000 x *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 [88].

2.9 Satistics

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 Figure 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 [51] and LBA9402 [54]. Fenugreek seedlings were infected following the procedure outlined in the corresponding methods section (summarized in Figure 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 Figure 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 (Figure 2A, arrowheads) and the number of explants with roots increased until 21 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 0-5% 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 (Figure 2B,C) or in liquid medium (Figure 2D). Roots grown on solid medium displayed growth rates of up to 1 cm/day (Figure 2B,

arrowheads), indicating that this method can be used to achieve rapid accumulation of hairy root biomass.

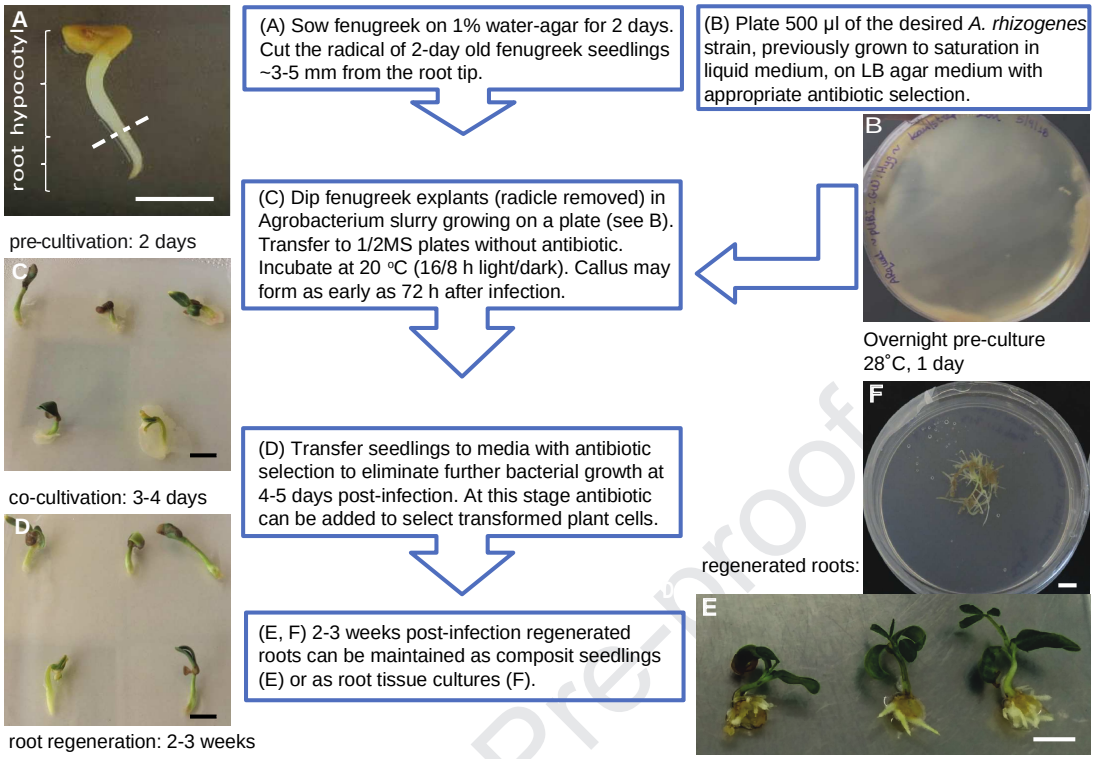

Figure 1: Protocol flowchart describing the procedure for fenugreek hairy-root transformation. The dashed line indicates the recommended point for radicle excision. Scale bars in all images are 1 cm.

Table 1: Results of fenugreek hairy root transformation using two different *A. rhizogenes* strains[1]

ARqua1[1]	Experiment 1			Experiment 2		
Days	Callus Formation	Root regeneration	Transformation efficiency[2]	Callus Formation	Root regeneration	Transformation efficiency[2]
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[1]	Experiment 1			Experiment 2		
Days	Callus Formation	Root regeneration	Transformation efficiency[2]	Callus Formation	Root regeneration	Transformation efficiency[2]
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	

[1]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.

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

- 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.

Figure 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.

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 Figure 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 Figure S2D, 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. 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 [89]. 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 [89]. 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 Figure 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 utilise polyubiquitin-derived or other endogenous housekeeping gene promoters from the target or closely related plant species [90]. Thus, we chose the *Lotus japonicus Ubiquitin1* promoter, which has been previously characterized and performs well in binary vectors used to transform *Lotus japonicus* [72].

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 Figure 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.

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

Experimental replicate	**1**	**2**	**1**	**2**
A. rhizogenes strain[1]	ARqua1 (p*Lj*Ubq::eGFP)		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[2]	50.0	57.7	-	-

[1]*Agrobacterium rhizogenes* ARqua1 transformed with p*Lj*UBQ::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.

[2]Percent of individual regenerated transformed hairy roots expressing GFP (transformation efficiency).

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 roods that was transformed based on PCR screening) was 76.4 ± 4.3%, with an overall transformation rate (the percentage of infected explants that led with transformed regenerated roots) reaching 28.3 ± 6.6%. Conversely, the transformation efficiency based on *EGFP* fluorescence was 53.8 ± 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 (*i.e.*, the percentage of infected explants that led to regenerated GFP-expressing roots) was 15.8 ± 5.9%, over both experiments (Table 2, Figure 3A-D, and Supporting Figure S4). Within transformed roots, strong GFP expression was detectable in all cell types, including: epidermal and root hair, cortical cell (Figure 3E-G), vascular bundle and root tip/cap and detached epidermal cells (Supporting Figure3 F-H). In individual cells, eGFP localized primarily to the cytosol and nucleus (Figure 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 [91]. 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.

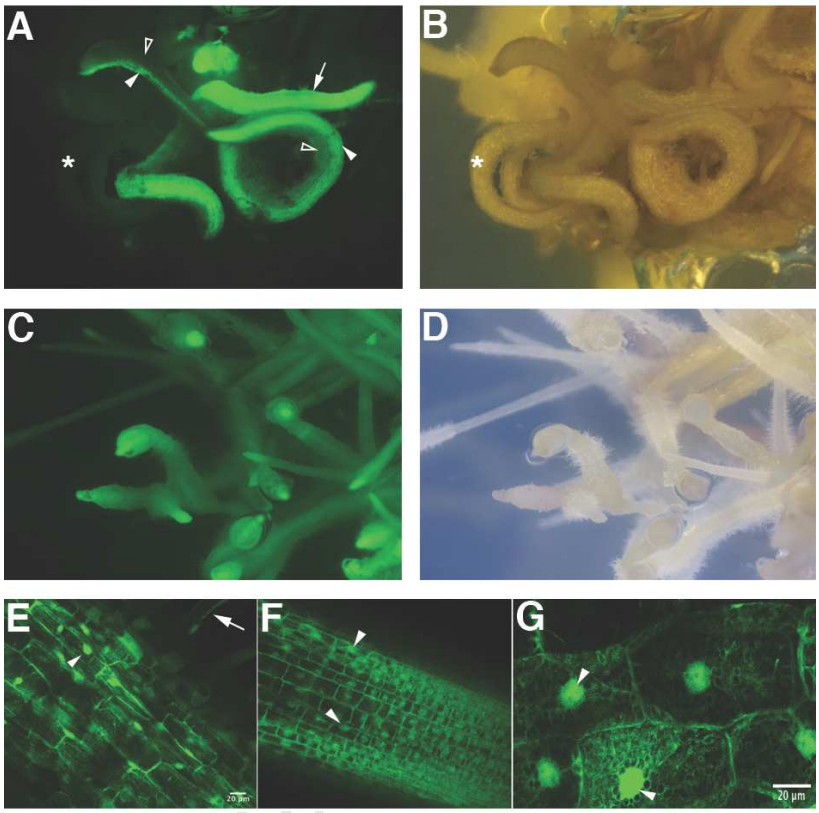

Figure 3: GFP fluorescence in regenerated fenugreek hairy roots. A) GFP and B) bright field images of regenerated hairy roots grown without selection three weeks after infection with the *A. rhizogenes* ARqua1 p*Lj*UB:GFP strain. Arrowheads and arrows in A indicate segments of chimeric regenerated hairy 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 *Lj*UBQ1 promoter. Nucleus-like structures (arrowheads) and root hair fluorescence (arrow) are indicated.

Although many of the regenerated roots uniformly express GFP (Figure 3A, arrow), some roots do not fluoresce at all (Figure 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 (Figure 3A, white arrowheads) while the other half does not (Figure 3A, open arrowheads). In some extreme cases, only a few cells in a regenerated root display fluorescence (Supporting Figure 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 [92, 93]. Even though the p*Lj*UBQ1::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 Figure 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* [73]. 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 *MtTSAR*1 and *MtTSAR*2 coding sequences from *Medicago truncatula* cloned independently under the control of the *LjUBQ1* promoter (*pUB::TSAR1/2::Hyg*, Supporting Figure 1) 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 Figure S6). Roots

identified in this manner were screened for expression of *MtTSAR*1 or *MtTSAR*2 using qPCR, and multiple individually transformed roots expressing these transcription factors were identified (Figure 4A, B). As expected, there was no detectable expression of either *MtTSAR1* or *MtTSAR2* in control samples. However, 66.7% (8/12 plants) of *MtTSAR*1-transformed plants and 42% (5/12 plants) of *MtTSAR*2 transformed plants showed heterologous expression of the corresponding *TSAR1* and *TSAR2* genes compared to the control samples (Figure 4A, B, asterisks).

Since we confirmed heterologous expression of *MtTSAR*1 and *MtTSAR*2 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 *MtTSAR*1 and *MtTSAR*2 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 [73, 94, 95]. We found that 87.5% (7/8) of plant lines expressing *MtTSAR*1 (Figure 4C) have reduced *HMGR* expression levels. Interestingly, 50% of lines (3/6) expressing the *MtTSAR*2 transcription factor (Figure 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 *MtTSAR*s to affect fenugreek endogenous gene expression. Both *MtTSAR*1 and *MtTSAR*2 strongly suppress CAS transcript expression in *Medicago* [73]. This was also the case for all of the *MtTSAR*1-expressing fenugreek lines, in which *CAS* expression was strongly suppressed (Figure 4E). However, *MtTSAR*2 had a minimal effect on fenugreek *CAS* expression with only 1/6 hairy root lines showing reduced *CAS* expression (Figure 4F). Additionally, *Mt*TSAR1 and *Mt*TSAR2 heterologous expression levels do not correlate either with HMGR or CAS (Supporting Table S3A).

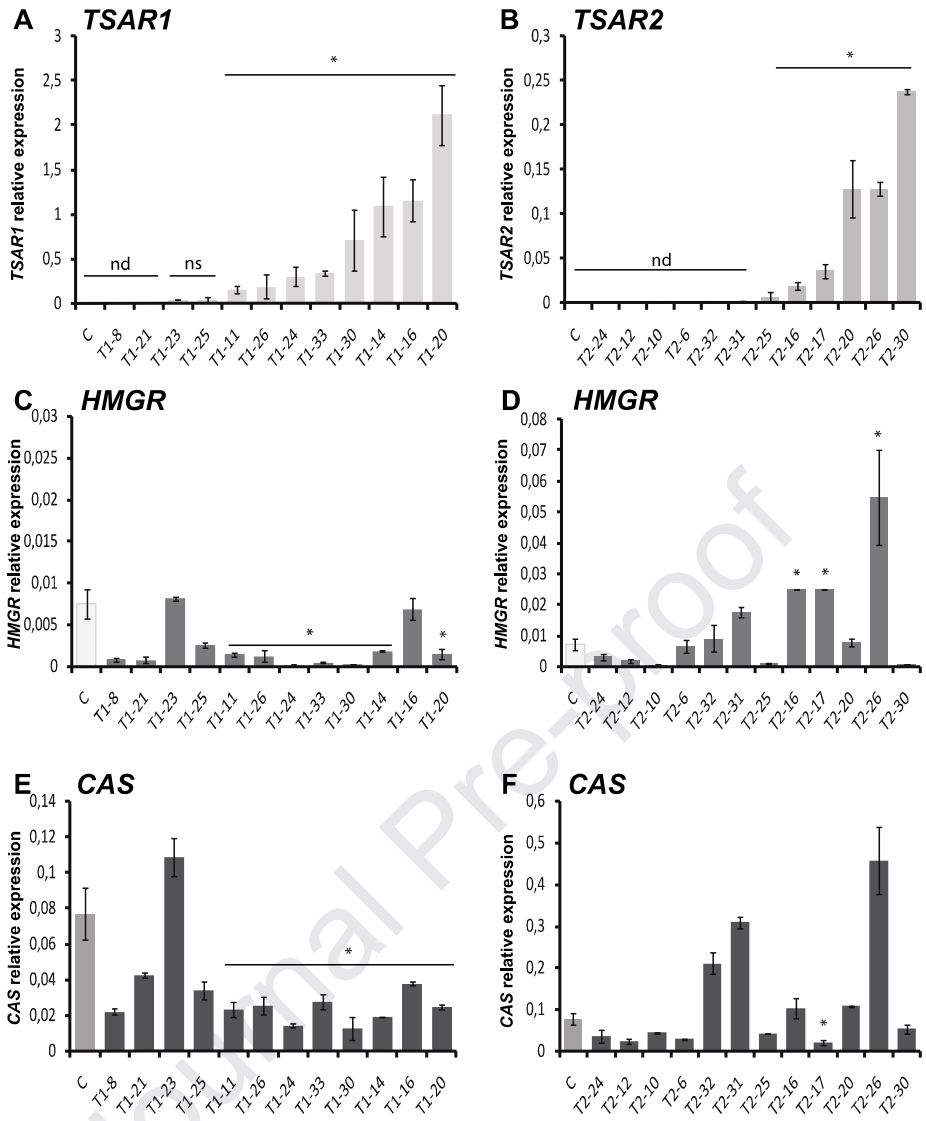

Figure 4: Heterologous expression of *MtTSAR1* and *MtTSAR2* in fenugreek hairy roots and the effect on HMGR and CAS transcript levels. Relative transcript expression estimated by qRT-PCR in control (empty vector) compared to A, C, and E) *TSAR1*-expressing and B, D, and F) *TSAR2*-expressing hairy roots; 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 ± SD of two technical replicates for each individual hairy root. Values for the control are the average±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.

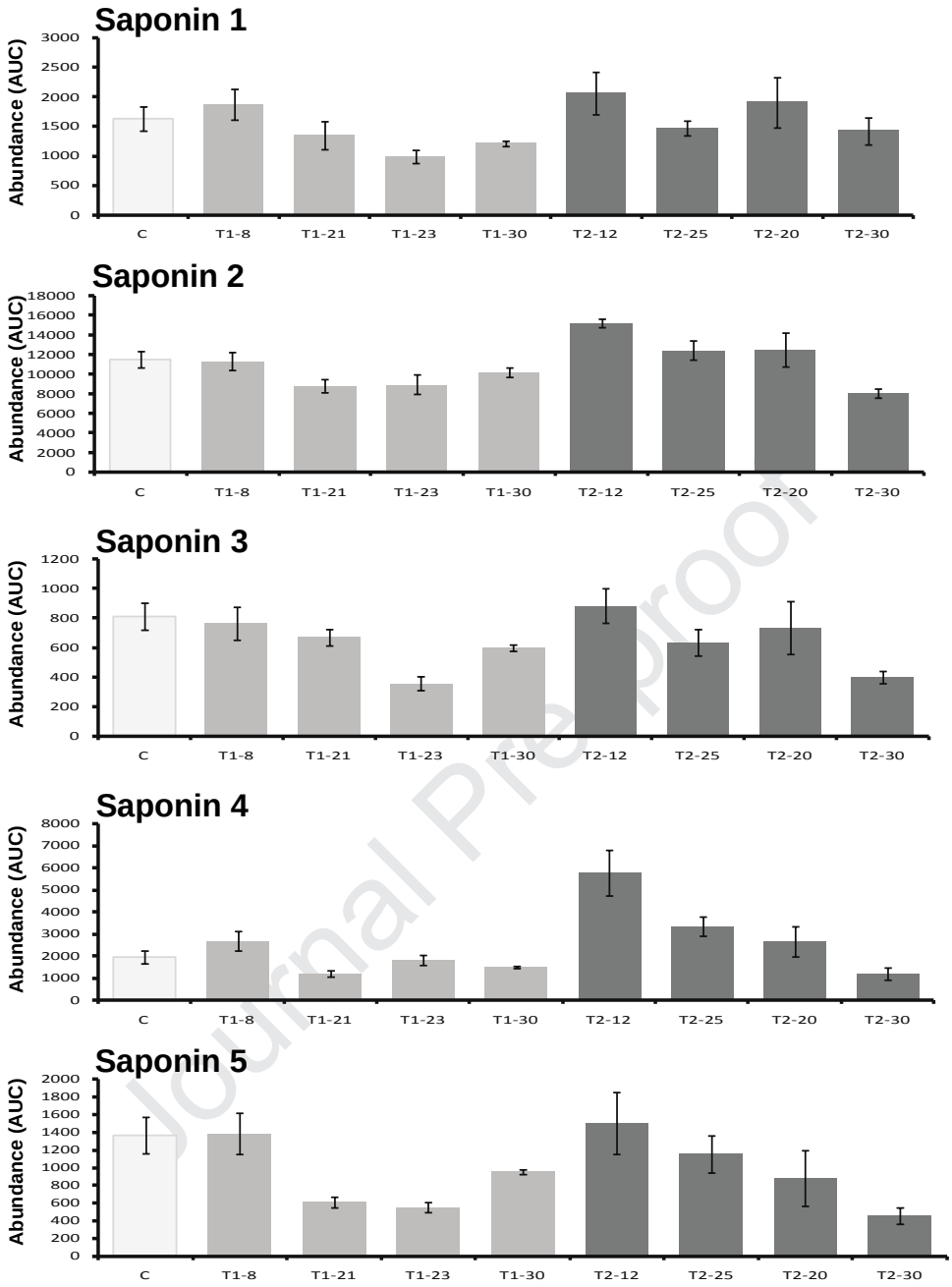

Figure 5: Relative abundance of five representative saponins in Fenugreek hairy roots expressing the heterologous *MtTSAR1* and *MtTSAR2* transcription factors. Values shown are the average ± SEM of at least three technical replicates per individual line. Values for the control are the average ± SEM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

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 (Figure 5), nor was there an observed increase in any saponin-like compounds identified during this analysis (not shown). Thus, while the heterologous expression of *MtTSAR*1 and *MtTSAR*2 in fenugreek does alter the transcription of endogenous saponin biosynthesis genes, we did not observe a major 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 *MtTSAR*s in fenugreek we transformed *Lotus japonicus* hairy roots with the same *MtTSAR*1 and *MtTSAR*2 expression constructs (Supporting figure S7A, B). Interestingly, *MtTSAR*1 did not affect *Lj*HMGR2 and only induced *Lj*HMGR1 and *Lj*HMGR3 in one of three lines (Supporting Figure S7C, D). However, *MtTSAR1* expression showed a significant positive correlation with *LjHMGR1* and *LjHMGR3* expression levels. On the other hand, plants expressing *MtTSAR*2 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 *LjHMGR*s (Supporting Table S3B). This suggests that the *MtTSAR*s 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 [48, 96]. 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 [97]. 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 specialized 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 [52]. In the second case [70], researchers used a particularly aggressive agropine-type *A. rhizogenes* strain K599 [53] to achieve a reported transformation efficiency between 70-80% 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* [51] 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 [98]. 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* [54].

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 [72] 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 [89]. This indicates very low activity of the CaMV35S promoter in fenugreek, similar to *Lotus* [72] and rice [90, 99]. 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 [100], and moreover is reportedly silenced in sections of nodules and conceivably other tissues in *Medicago truncatula* [101]. 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 *MtTSAR*1 and *MtTSAR*2 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 [73]. This correspondingly led to an increase in hemolytic or

non-hemolytic triterpene saponin levels, depending on which TSAR transcription factor was overexpressed [73]. A similar alteration in saponin biosynthesis gene transcript levels and an increase in non-hemolytic saponin accumulation was observed when the *Cr*BIS1 (bHLH irridoid synthesis 1) from *Catharanthus roseus* was heterologously expressed in *Medicago* hairy roots [102]. We did not observe a significant alteration in saponins in fenugreek roots expressing *Mt*TSAR1 and *Mt*TSAR2. 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 (Figure 3A, arrowheads). These mosaic roots will show TSAR transcript accumulation greater than control roots (in which they are non-detectable, Figure 4A,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 *Cr*BIS1 expression in *M. truncatula* hairy roots was the *MtHMGR1* gene [73, 102]. 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 *MtTSAR*2 in fenugreek. This was comparable to the effects of heterologous *MtTSAR1* and *MtTSAR2* expression in *Lotus japonicus* hairy roots, wherein *MtTSAR*2 consistently induced the expression levels of the endogenous *LjHMGR2* gene, while *MtTSAR*1 caused an induction of *LjHMGR1* and *LjHMGR3* in only one of three roots (Supporting figure 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 *MtTSAR*s 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 [103]. 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 [73]. 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 [104]. Conserved regions of the basic helix-loop-helix (bHLH) structure of TSARs, which are normally responsible for interaction with other proteins [105, 106], 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 *MtTSAR*1 and *MtTSAR*2 (Supporting Figure 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.

5. Competing Interests

The authors declare no conflict of interest

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7. 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.

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Declaration of interests

V 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: